Sequestration of heavy metals and radionuclides in ectomycorrhiza

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Abbreviations and symbols

AM	arbuscular mycorrhiza
AMD	acid mine drainage
AQP	aquaporin
AZA	acetazolamide
C	crown
C+S	crown and stem
ECM	ectomycorrhiza
GD	guttation droplets
GSH	reduced glutathione
GST	glutathione S-transferase
GTT	fungal glutathione transferase
HM	heavy metal
ITS	internal transcribed spacer
M	mycelium
MIP	major intrinsic protein
PCR	polymerase chain reaction
R	root of tree
R+M	root and mycelium
Ra	radionuclide(s)
RT	room temperature
S	stem of tree
U	unit
wt	wild type

Summary

Secondarly mineral enriched fields, like those found in post-mining areas, are potentially economically interesting for the extraction of valuable elements. The involvement of microorganisms such as fungi present in the environment take part in this process of element re-distribution.

Ectomycorrhiza (ECM) was studied concerning metal and radionuclide distribution within the system soil-ECM. The development of the ECM tree partners *Picea abies* and *Pinus sylvestris* was studied in relation to a symbiosis with the early colonizer ectomycorrhizal (ECM) fungi *Paxillus involutus* and *Pisolithus tinctorius*, as well as the late colonizer fungus *Tricholoma vaccinum*. With pot experiments, the influence of ECM on metal distribution in soil was analyzed.

High bioconcentration factors (BCF) associated with metal enrichment in the fungal cell was found to correlate with low glutathione S-transferase (GST) activities. While early colonizers showed higher GST activity in the mycelium as well as in mycorrhizal roots, the late colonizer *T. vaccinum* had lower or even lacking GST activity; the mycorrhizal roots as well featured a lower GST activity.

Another measure for dealing with metals could be identified with guttation droplets that may detoxify high metal loads. Basidiomycetes excrete secondary metabolites and nutrients, like sugars or amino acids, *via* guttation. With this guttation fluid, nutrients can be distributed to potential tree partners, attract other ECM fungi or soil organisms, or promote further dispersal of the extramatrical mycelium. Additionally, high Pb values were measured in guttation droplets after cultivation in Pb supplemented media, which shows detoxification for survival under harsh environmental conditions. An involvement of aquaporin proteins forming water channels in the membrane in guttation could be shown. The transfer of water as well as gases or soluble substances can be inhibited by acetazolamide and silver ions which led to less guttation and altered element contents in the guttation fluid.

Here, fungi could be shown to determine element concentrations in their host plant, and keep homeostasis within their cells as well as excreting metals with guttation. Thus, the role of fungi in element cycling can be used for gaining valuable elements for biologically supported mining operations.

Zusammenfassung

Insbesondere Bergbaufolgelandschaften wahren große Mengen wertvoller Mineralreservoirs, welche durch Abbautätigkeiten der Erze angereichert wurden. Durch die vermehrte Herstellung und Nutzung von Kommunikations- und Technikprodukten steigt zeitgleich die Nachfrage nach begrenzten oder bereits resourcenerschöpften Mineralien. Dies birgt in diesem Zusammenhang eine große wirtschaftliche Bedeutung bezüglich des Rohstoffschatzes von Bergbaufolgelandschaften.

In dieser Arbeit wurde die Aktivität von Ektomykorrhizapilzen für die Verteilung von Metallen und Radionukliden im System Boden-Mykorrhiza-Pflanze untersucht. Der Einfluss der Mykorrhizapilze auf die Entwicklung der Baumpartner *Picea abies* und *Pinus sylvestris* sowie ihre Sukzession wurde anhand der Frühbesiedlerpilze *Paxillus involutus* und *Pisolithus tinctorius* sowie dem Spätbesiedlerpilz *Tricholoma vaccinum* charakterisiert. In Topfversuchen konnte gezeigt werden, dass die Ektomykorrhiza (ECM) zu Änderungen der Elementkonzentrationen im Boden führen kann. Dabei spielt die Akkumulation von Elementen eine wesentliche Rolle.

Hohe Biokonzentrationsfaktoren (BCF) führen zu hohen intrazellulären Metallkonzentrationen. Diese waren dennoch mit einer niedrigen Gluthation S-Transferase-Aktivität gekoppelt. Dabei zeigten die Frühbesiedler höhere Aktivitäten im Myzel und in den symbiontischen Baumwurzeln, während beim Spätbesiedler *T. vaccinum* deutlich geringere oder fehlende GST-Aktivität beobachtet wurde, die in der Symbiose noch niedriger lag.

Ein weiterer Prozess, der der Ausscheidung von Sekundärmetaboliten und Nährstoffen wie Zucker oder Aminosäuren, aber auch Metallen dient, ist die Guttation. So konnten hohe Blei-Werte in Guttationstropfen gemessen werden, wenn der Pilz auf Blei-haltigen Medien kultiviert wurde. Dieser Weg der Entgiftung kann dem Pilz das Überleben unter extremen Bedingungen erleichtern. Andererseits kann der Mykorrhizapilz so aber auch potentielle Baumpartner mit Nährstoffen versorgen, andere ECM-Pilze und Bodenorganismen anlocken oder sogar die Ausbreitung des eigenen Myzels fördern. Eine Beteiligung von Aquaporinen an der Guttation konnte gezeigt werden. Die multifunktionalen, membranintegrierten proteinogenen Poren aus Aquaporin können neben Wasser auch Gase und gelöste Stoffe transferieren. Da die Aquaporin-Inhibitoren Acetazolamid und Silber eine Reduktion der Guttationstropfen-Bildung am pilzlichen Myzel bewirken, konnte ein Einfluss auf die in der Guttationsflüssigkeit enthaltenen Elemente gezeigt werden.

Im Rahmen dieser Arbeit konnte der Effekt von Mykorrhizapilzen auf Elementzyklen beobachtet werden, dass Mikroorganismen wie Pilze, Elemente intrazellulär festlegen und extrazellulär transferieren können. Somit können sekundär angereicherte Elemente aus ihren mineralischen Bindungen, neben den aktuellen Technologien, auch auf biologischem Weg herausgelöst und damit verfügbar gemacht werden.

1. Introduction

1.1. Soil microbe-root interactions

Especially soil-microbes, play an important role with regard to plant productivity and diversity within terrestrial ecosystems (van der Heijden *et al*. 2008). Long-lasting effects of environmental impacts immensely influence the soil and, in consequence, the essential elements like carbon, nitrogen or phosphorus are bound in the soil while the concentrations vary depending on the ecosystem (Lal 2015). Therefore soil is not only ground but much more. It functions as archive, multifaceted accumulator, acreage for agriculture, source for natural resources, delivers energy and provides valueable living space for organisms. Within one hectar space, for example, there exist 15 tons of living soil organisms; bacteria, fungi, algae, invertebrates like worms, insects, larvae and others. In general the proportion of fungi thereby has an amount of around 100 billion (Ehlers 2015).

The basic assumptions of interactions are related to the natural relationships between organisms and their environment (Walter and Hengeveld 2000). In ecological contexts there are beneficial and antagonistic interactions. Both are important drivers of evolution. There are different kinds of beneficial interactions that can occur within or between species. Examples are parasitism, known as altruistic behavior, or commensalism, which is beneficial for only one partner. Prominent interplays that benefit both partners are cooperation and mutualism, especially the symbiosis ectomycorrhiza.

As simple form of coexistence fungal-bacteria interactions are described *via* hyphal attachment with larger and widespread infection areas (Schreres and Krom 2016). Antagonistic effects in fungus-bacteria interactions are documented *via* reduced hyphal growth within biofilms affected by bacteria *Staphylococcus aureus* (Ramirez Granillo *et al*. 2015). Plant-fungus interactions, like arbuscular mycorrhiza of *Glomus intraradices* and several herbaceous plants, have evolved over a long period (Smith and Read 2008). Interactions between microbial activity of bacteria, spore germination and hyphal growth indicate a removal of toxins or germination inhibitors. As a result an improved migration of the hyphal system of the fungus is shown (Le Tacon *et al*. 1983). Fungal hyphae are used as paths or fungal highways and are known for bacterial migration (Warmink *et al*. 2011). Bacterial helper effects are described as stimulation (Founoune *et al*. 2002, Labbe *et al*. 2014, Kurth *et al*. 2015) or assistance of mycorrhiza formation (Frey-Klett *et al*. 2007) under different climate conditions. The spreading of fungal mycelium interactions can be

determined by either active or passive processes. On the one hand there is an active excretion of substances, like guttation, secondary metabolites or volatiles. On the other hand it is passively driven by factors like soil structure, pH, temperature or humidity (Mahmood 2003).

The mutualistic association and interaction ectomycorrhiza (ECM) is an obligate mandatory eu-symbiosis with a long evolutionary adjustment (Bidartondo *et al*. 2011). The symbiosis between the interspecific organisms tree and fungus can be described as life improving beneficial for both, the plant and the fungal partner. Mineral aggregations with fungal hyphae as special kind of abiotic interactions benefit the settlement of a broad range of soil types (Colpaert *et al*. 2011). In particular the abiotic interactions hyphae-soil particles or minerals are interesting because of their high potentials. Concerning biosorption effects different interactions between elements and organic substances are described by Fomina and Gadd (2014). Basen on the physico-chemical processes of adsorption, ion-exchange and complexation (Gadd 2009), precipitation of metals in minerals can interact with hyphal organic material or active processes of fungal metabolism. Soil water and light is clearly affecting temperature and is therefore an important driver for element-hypha interaction. Higher temperature can enhance sorptive effects (Fomina and Gadd 2014) and faster tree root colonization of ECM fungi (Erland and Finlay 1992). Furthermore high metal binding capacity of ECM fungal mycelium (Tobin *et al*. 1990, Berthelin *et al*. 1993) with subsequent accumulation (Berthelsen *et al*. 1995, Marschner *et al*. 1998) is well known from literature. The mutualistic symbiotical interaction ECM with focus on its fungal partner in regard to environmental abiotic features will be highlighted in this work. In this context I will also focus on the influence of contaminations or life hamper conditions.

1.2. Ectomycorrhiza

Mycorrhizas are tight associations between fungal mycelium and plant roots or other underground organs and help to provide mineral nutrients. Through the hyphal network nutrients are transported to the plant roots (Deacon 2006). The term “fungus root” first defined by Frank (1885) thereby refers to the physiological function and several fungal associations with plant roots. Mycorrhiza in general occurs in around 80% of terrestrial vascular plants within angiosperms and gymnosperms. Arbuscular mycorrhiza (AM) is widespread on crop and herbaceous plants. Physiologically the AM enters the cortical cells of the plant and develops fungal arbuscules and storage vesicles inside or between plant cells (Smith and Read 2008).

In particular ECM or ectotrophic (outside-feeding) mycorrhiza is confronted with two potential life styles. They form either symbiosis with plant roots but can also facultatively exist in the soil as saprobes (Nehls *et al*. 2009). Characteristically ECM can be found in lignified, woody plants like trees and shrubs, especially of the families Pinaceae, Fagaceae, Betulaceae and Myrtaceae. The importance of ECM fungi lies in their outstanding effects on seedling establishment and tree development (Tedersoo *et al*. 2010). Their fungal tissue intercellularily enters the plants epidermal and cortical cells to form a "Hartig`net" to exchange nutrients, water and elements (Smith and Read 2008). Externally the plant roots are enclosed by the hyphal mantle, which contains extraradical hyphal elements and replaces the function of the root hairs. ECM extramatrical mycelium has much lesser diameter and therefore advantages in reaching small, dense and packed soil structures compared to the root hairs. In addition rhizomorphs, the fungal aggregation of mycelia cord structures, provide the plant with nutrients, minerals and water over a large distance (Simard *et al*. 2002). Characteristical ectomycorrhiza short roots formed by a sheath of fungal tissue are shown in Fig. 1.

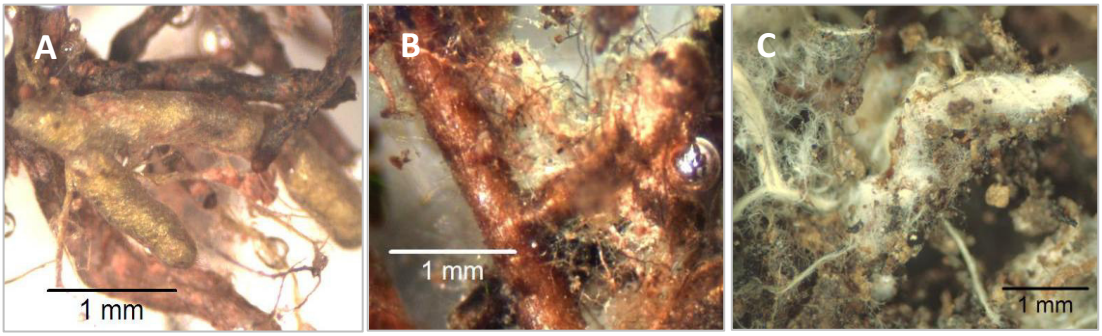

Fig. 1: **Short root**s of **A** *Pisolithus tinctorius* from *Betula pendula*, collected at a heap, Ronneburg, Germany. **B** *Paxillus involutus* from established ca. 50 years old mixed forest, nearby Jena, Germany. **C** *Tricholoma vaccinum* with *Picea abies* from established needle tree forest Münchenrodaer Grund nearby Jena, Germany.

Mainly eukaryotic fungi of Glomeromycota, Ascomycota and Basidiomycota are able to form mycorrhizae. Hyphal filaments grow apical, branch behind tips and form spacious, interconnected and branched networks. Main carbon sources (C-sources) for saprotrophic living are located in dead organisms or litter. ECM fungi benefit from organic compounds, obtained by the plant, and practice the heterotrophic, chemo-organotroph, living by taking up mineral nutrients from soil. These compounds are used as energy source and for cellular synthesis to form cell wall components like chitin or glucans (Deacon 2006, Nehls *et al*.

2009). C-sources delivered by plants are amino acids, carboxylic acids and soluble sugars, like mannitol, arabitol and erythriol (Harley 1989, Nehls *et al*. 2008) while hexoses are the main C-sources (Smith and Read 2008). One important benefit for enhanced plant growth is due to the fungal tissue like rhizomorphs or hyphal cords that provide the translocation of nutrients and water in hyphae. They are synthesizing vitamins, auxins, gibberellines and amino acids (Smith and Read 2008).

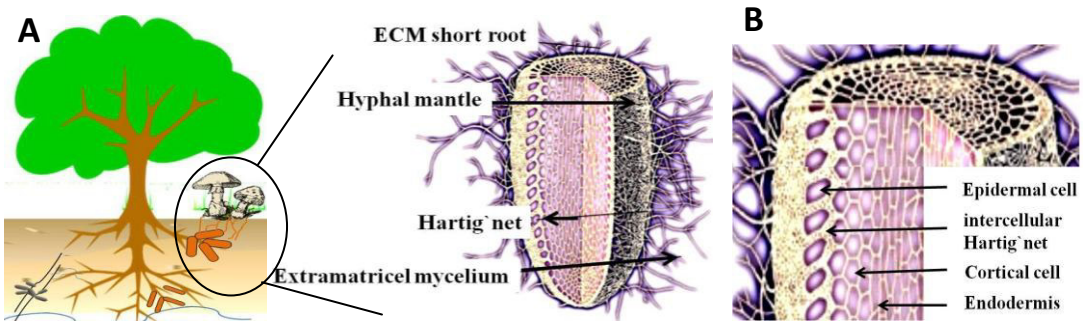

Fig. 2: **ECM short root with ectomycorrhiza features**. **A** extramatricle mycelium, hyphal mantle and Hartig`net. **B** Hartig`net - region of nutrient, water, minerals and ligands exchange between two ectomycorrhizal partner. (modified after Gagné 2005).

The characteristic mycelia features of ECM short roots have conspicuous ecological relevance. ECM short roots tend to localize mainly in the mineral soil. By generating fungal rhizomorphs the ECM explores the overlying litter that serve as nutrient resources (Smith and Read 2008). Agerer (2001) defined different exploration types of ECM inspired by short root or morphotype features following different strategies. Based on the development and expression of the extramatrical mycelium (Fig. 2) and the presence or absence of rhizomorphes the important functions of ECM is shown by tree growth promotion and substrate stabilization. Rhizomorphs can interconnect trees over large distances, as described by Schramm (1966) for *Pisolithus tinctorius* and pine over 42 m. The ECM fungi *P. tinctorius* and *P. involutus* belong to the long-distance exploration type with few, highly differentiated rhizomorphs and smooth short roots. *T. vaccinum* can belong either to the stated or the medium-distance exploration type with infrequently rhizomorphs and thick but few emanating hyphae (Agerer 2001). So the morphology of the ECM short roots indicates the ecological life style of the fungus. The function of the ECM is densely connected to the qualities of the soil and the established exploration type. By the help of the exploration types a cost/benefit quantification of the tree-fungus symbiosis can be done (Weigt *et al*. 2012).

1.3. Early and late colonizing fungi

In relation to ectomycorrhizal fungi a faster or slower spore germination, with distinction in early and late colonizer, seems to be advisably. *Hebeloma* spec., *Laccaria* spec., *Thelephora* spec., *Pisolithus tinctorius* or *Paxillus involutus*, e.g., are usually the first species that germinate and form fruiting bodies, around young trees and are called early colonizers (Smith and Read 2008). While in the vegetation dynamic a change of the structure and composition of the species can be found. These vegetation dynamics can be called succession, - depending on their scales there are known primary and secondary succession. (Schulze *et al*. 2002). In primary succession woody plants rely on ECM fungi partner for establishment (Nara 2006a) because of their less adaptive option for tolerance of toxic elements and long sensitive reproductive life cycles (Meharg and Cairney 2000). In primary succession ecosystems with lower requirement to carbon supply and host specificity ECM form short roots over the whole plant root system. Whereas late colonizer like *Russula* spec., *Lactarius* spec. or *Tricholoma* spec. prefer more established sites like secondary succession ecosystems. They are more likely to select plant hosts with an increased exchange rate of carbon and prefer to form short roots with older plant root parts (Smith and Read 2008).

Both, early and late colonizers, have the ability to dispose about the community interaction of overgrowing (Danielson and Visser 1989). So in distal parts of the branched roots and after dormancy (Fleming 1985) an overgrowth associated with replacement of the species can appear. This can ensure a constant supply of nutrients for the plant partner. The permanent association of the plant host with fungal partners and potentially plant vitality is ensured by fungal support (Molina *et al.* 1992). In addition ectomycorrhizal fungi show different affinities and compatibilities for host plants. If the partners fit less, the ECM tissue formation rarely induces establishment of fungal-plant morphological structures and shows no functional ectomycorrhizal structures. Therefore in cases of incompatibility the hyphal mantle is weakly developed, which was studied with three selected early and late colonizer ECM fungi by Pereira *et al*. (2005) and within late colonizers of genus *Tricholoma* (Krause 2005).

My studies are about two early and one late colonizer ECM fungus: *Pisolithus tinctorius* (Basidiomycota, Agaricomycetes, Sclerodermataceae) is an early colonizer ECM fungus with clear preference for heaps and disturbed areas that is evidently their ecological niche (Marx 1977, Medve and Shan 1982, Fig. 3). Also *Paxillus involutus* (Basidiomycota, Agaricomycetes, Paxillaceae) can be described as early colonizer fungus with wide distribution in the Northern hemisphere (Wallander and Söderström 1999, Fig. 3). Both

species have a markedly broad host specificity, low demand for plant derived carbon. Therefore they are optimally adapted to fields of primary succession or disturbed substrates. *Tricholoma vaccinum* (Basidiomycetes, Agaricomycetes, Tricholomataceae) is a late colonizer ECM fungus with host specificity to coniferous trees like *Picea abies* but also *Pinus* spec (Fig. 3). Therefore it is optimally adapted on established forest-systems and boreal up to sub-boreal climate (Singer 1986).

Fig. 3: **ECM fruiting bodies**. **A**: *Pisolithus tinctorius* ECM symbiosis with *Quercus robur*, **B**: *Paxillus involutus* ECM symbiosis with *Picea abies*, both Ronneburg, Germany. **C**: *Tricholoma vaccinum* ECM symbiosis with *Picea abies*, Münchenrodaer Grund Jena, Germany.

1.4. Detoxification mechanisms

The cellular detoxification mechanisms and metal homeostasis can be seen as an answer to stress metal tolerance, with associated mechanisms of metal mobilization and immobilization, can be found as an adaptation to high metal concentrations in the soil. The intracellular response and detoxification mainly is achieved through reactions of the primary metabolism. These reactions can trigger defence reactions like conjugation of the xenobiotics, or the secretion of the conjugates into vacuoles or with secretory vesicles (Coleman *et al*. 1997, Fig. 4). Extracellular chelation by excretion of ligands, precipitation, cell-wall binding and enhanced efflux are other potential detoxification mechanisms.

1.4.1. Toxic elements that potentially interrupt fungal cell metabolism

To determine element concentrations in fungal tissue, bioconcentration factors (BCF) are used. The BCF values are non-dimensional indices for bioconcentration of elements in organic tissue and provids to determine accumulations of elements inside organisms compared to the environmental surrounding. Based on the BCF`s an assessment and

classification of fungal or plants ability to exclude or accumulate elements, in compartments relatively to bioavailable element concentrations of the ambient environment, can be done (Bahadir *et al.* 1995). While the ability of organisms to bioaccumulate substances, the exclusion of elements requires chemical or mechanical barriers arranged by the individuals. This implies the option of uptake of substances by cell surfaces and provides the opportunity to quantify bioconcentrations.

Values < 1 indicate exclusion and values > 1 indicate accumulation of the particular element. Per definition the BCF value is the ratio between the concentration of chemicals in organism and that in the surrounding medium. It yields after the following term:

(1) $$\mathbf{BCF} = \frac{\textbf{Concentration of elements organic tissue}}{\textbf{Bioavailable concentration of elements in soil}}$$

BCF > 1 Element concentration in organic tissue **higher** than bioavailable in soil (accumulation)

BCF = 1 Element concentration in organic tissue **equals** the bioavailable in soil (no accumulation or exclusion)

BCF < 1 Element concentration in organic tissue **lower** than bioavailable in soil (exclusion)

In this context several studies of BCFs for natural grown and edible fungi can be found (e.g. Kalac and Svoboda 2000, Svoboda *et al.* 2000). Documented BCFs of different basidiomycete fungi like *Agaricus spec.* (Sarikurkcu *et al.* 2011), *Telephora terrestris* or *Lactarius spec.* (Schindler *et al.* 2012) range from 50 to 300. So the BCFs indicate high accumulation and range their respective tolerance levels. The BCF distribution pattern for Cs^+, e.g., follows this order: hat skin > lamella > hat > stem. The hat skin implies the highest and the stem of the fungal fruiting body the lowest elemental Cs^+ content (Sporleder 2011). The highest Cs^+ (59%) concentrations of radioactive ^{137}Cs was found in lamellae (Heinrich 1993), after hat tissue and stem (van Elteren *et al.* 1997, Baeza *et al.* 2006). Especially on locations with stronger elemental concentrations in the soil, fungal fruiting bodies show larger elemental loads (Turnau *et al.* 1994). But which elements are accumulated without importance for growth or reproduction of fungi?

The distinction in essential and non-essential elements shows therefore directions. After Gadd (1993) essential elements are Ca, Co, Cu, Fe, K, Na, Mg, Mn, Ni and Zn with cell physiological relevance in biochemical reactions, and non-essential elements are Ag, Al, Au, Cd, Cs, Hg, Pb and Rb without cell physiological relevance in biochemical reactions. But essential elements in high concentrations can act toxic too. Consequences for organisms in this context can be enzymatic inhibition of reactions or growth stimulation (Gadd 2007). Generally very specific effects on organisms can result. The alternative distinction of non-

essential, in high concentrations toxic elements, can be the termination: heavy metals. As toxic elements they are differently characterized by their physical (density, atomic weight, atomic number) and chemical (chemical properties or toxicity) qualities. Another range of non-essential harmful elements are radionuclides. As collective term for all nuclides, they are characterized by their radioactivity.

To chose the elements which are used in investigations of this study, I would like to focus on the (heavy) metals Cd, Ni, Pb and the radionuclides Cs and Sr.

Cadmium (Cd), with a physical density of 8.69 g/cm³, exists elemental as transitional stage and remains only chemical bounded. Cd is a cumulation toxin, industrial waste or by-product in metal processing industry, like Ni-extraction, and appears in slurries. By burning of fossil fuel and waste, gaseous release of Cd occurs into the atmosphere. High loaded dust inclusion induces an enrichment of Cd in liver and kidney. By blood bound transport, Cd attains to the liver and induces and bounds there to cysteine-rich metallothionein proteins. Subsequently the cadmium-metallothionein-complex reaches and accumulates in kidneys. But also the thyroid, pancreas, salivary glands and placenta enriches Cd. (Hartwig 2005). It acts as inhibitor of sulphydryl enzymes and has an affinity for cell ligands like hydroxyl, carboxyl, phosphatyl, cysteinyl and histidyl side chains of proteins (Dara 1997). Physiologic Cd^{2+} ions compete in bones with Ca^{2+} ions, as affector of Ca regulation in biological systems. Furthermore Cd can affect fertility and damages unborn children in the womb of mammals, it acts carcinogenic and mutagenic, is an endocrine disrupter as well as damages lung and fragile bones. (Degreave 1981, Salem *et al*. 2000). Bioconcentrationfactors (BCF) of sea fish amounts about 1.000 and even for phytoplankton around 10.000 are documented (Hartwig, 2005).

Nickel (Ni), with a physical density of 8.902 g/cm³, is in moderate concentrations an essential trace element especially for plants, as part of the urease. Several microorganisms, e.g. bacteria, are using Ni as part of the methyl-coenzyme-M-reductase. It`s resorbed in the gastrointestinal tract by passive diffusion. Nickel dust and aerosols are cancerogenic and can induce pneumonia, liver and kidney failure as well as general poisonous symptoms in higher amounts. (Hartwig, 2006). Furthermore Ni can act immunotoxic, neurotoxic and genotoxic, affects fertility and hair loss (Salem *et al*. 2000, Duda-Chodak and Baszcyk 2008, Das *et al*. 2008).

Lead (Pb), with a physical density of 11.3 g/cm³, is described as very toxic heavy metal with high probability for teratogenic effects. Pb isotopes represent final products in 3 of 4 natural radioactive decay series. Ingested aerosols, e.g. by lungs, are well absorbable. The

insidious uptake of Pb is very harmful. Pb binds in blood loosely onto erythrocytes and accumulates in bones, teeth and hair where it can substitutes Ca. A chronically exposition induces blocking of enzymes like free thiol-groups. Children and infants are specific sensitive endangered with regard to mental development and impaired development by reduction of intelligence, short-term memory loss, disabilities in learning and resulting coordination problems (Salem *et al*. 2000, Wuana and Okieimen 2011). WHO restrictions of preliminary bearable weekly inclusion amounts represent 25 µg/kg body weight for infants and children (WHO 2000). Under natural environmental conditions Pb is transferred into insoluble forms, aerobic in basic Pb carbonates and anaerobic in Pb sulphides. Pb-compounds adsorbs well with clay and humic substances, in the soil. Swallowing and inhalation is toxic, the adsorption by skin can interrupt synthesis of hemoglobin, it blocks enzymes and prevents Fe-assembly into hemoglobin molecules, furthermore it disturbs on this path the oxygen supply of somatic cells. (Hartmann-Schreier, 2006)

Cesium (Cs), with the physical density of 1.9 g/cm³, is a rare earth element and alkali metal. Next to the natural Cs isotope, ^{133}Cs, different artificial isotopes exists. Cs is a ignoble, highly reactive, explosive and highly flammable, metal. It resembles strongly in its properties the other alkali metals, especially K. In nuclear fission processes arising ^{137}Cs, is a very hazardous radioactive gamma ray. It is ingestable by food, especially fish, milk products, legumes, fruits, cereals and fungal fruiting bodies. Cs resorbs completely in the gastrointestinal tract. Cesium compounds are well water soluble. It has a long residence time in the soil and is easy available for plant roots. (Römpp 2002, Seilnacht 2015). Stable Cs, ^{133}Cs, has strongest effects to physiological processes of living organisms (Bystrzejewska-Piotrowska and Bazala 2008).

Strontium, with a physical density of 2.64 g/cm³, is an alkaline earth metal. Naturally occuring isotopes are ^{84}Sr, ^{86}Sr, ^{87}Sr and ^{88}Sr. Additionally artificial isotopes and isomers with short half-life periods exist, ^{77}Sr until ^{102}Sr. The beta ray ^{90}Sr deposits mainly in children marrow. Caused by chemical similarities with Ca, it reduces Ca transport and integrates into bones with long lasting irradiation effects. (Sitzmann 2007, Seilnacht 2015)

1.4.2. Mobilization and immobilization of elements

Microorganisms are able to influence and contribute in mobilization and immobilization of metal elements, in particular in soil compartments among soluble and

insoluble phases. Important mobilization processes for microorganism, especially fungi, are protonation (chemoorganotrophic leaching), chelation by means of metabolites and metallothioneins, siderophores and methylization, especially chemical transformation. All these processes are options to dissolute or desorb metals, radionuclides, metal compounds, minerals and organic or anorganic chemicals from exchangeable sites of organic matter or clay minerals, seen in Fig. 4 (Bellion *et al*. 2006). On the other hand are immobilization processes like sorption, transport and intracellular sequestration or precipitation. (Gadd 2004).

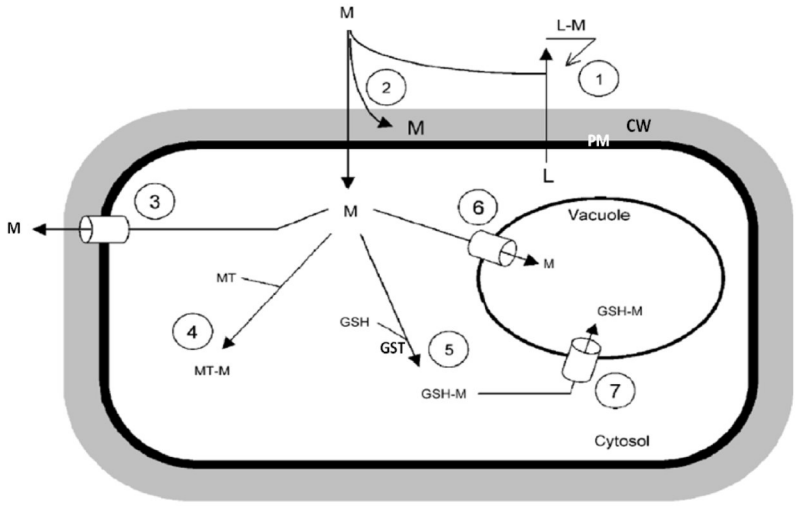

Fig. 4: **Cellular mechanisms involved in metal tolerance in ectomycorrhizal fungi**. L – ligand, M - metal-ion, L-M – ligand-metal-complex, MT – metallothionein, MT-M – metallothionein-metal-complex, GSH – glutathione, GSH-M – glutathione-metal-conjugation, PM – plasma membrane, CW – cell wall, GST – glutathione S-transferase. 1) extracellular chelation by excreted ligands; 2) cell-wall binding; 3) enhanced efflux; 4) intracellular chelation by metallothionein; 5) intracellular chelation by gluthathione; 6) subcellular compartmentation (vacuole or other internal compartments) and 7) vacuolar compartmentation of GSH-M-complex (slightly modified after Bellion *et al*. 2006).

By protonation (chemoorganotrophic leaching), the process within microorganisms can acidify their direct environment by proton efflux, a constant acidification can result in release of free metal ions, e.g. cations (Gadd 2004). Citrates and oxalate ions as well oxalic acids, can mobilize metal citrates and soluble oxalate complexes which bind furthermore to organic matter or clay minerals (Strasser *et al*. 1992, Gadd *et al*. 2014). Organic acids generally can corrode minerals and therewith strongly influence soil formation and biogenic chemical weathering (Gadd 1999). Chelation and on this way complexation is an important tool for mobilization effects on elements and bounding of metabolites. Low molecular weight molecules, like siderophores and resulting iron fixation, are prominent examples. (Gadd 2001). Siderophores in this case are highly specific iron (Fe(III)) ligands, but bind Mg, Mn, Cr (III) and radionuclides too (Birch and Bachofen 1990). In consequence solubilized

elements adsorb biomass or precipitate thereby. Further solubilization of metals and elements induces a removal of xenobiotics from solid matrices like soil, sediment, dumps and other industrial wastes. Chemical transformations or biomethylation occur under aerobic and anaerobic conditions for example within the elements Pb, Hg or As. Within this enzymatic transformation methyl groups are transferred to ions with change of solubility, volatility and toxicity of these afterwards methylated metal compounds. (Gadd 2004). Furthermore the mobilization of elements, metals or radionuclides can be induced by reduction and oxidation processes (Lovley 2000). For example by reduction of U(VI) to U(IV) the metal solubility decreases and results in an immobilization (Phillips *et al*. 1995). All these processes lead to release and decompensation of elements, metals, radionuclides or substances from organic matter, soil particles or living cells.

In general by the help of immobilization processes, cell external free ions are immobilized. By the process of sorption, or biosorption, organic and inorganic ions are adsorpted to biomass or exopolysaccharides by physicochemical mechanisms. This can be described as enrichment of one substance in the organic tissue or between two phases. Living cells influence biosorption through metabolic activities like changes of pH, E_h as well as the supply of organic or inorganic nutrients and metabolites. (Gadd 2004). Additionally biosorption is an important tool within development and crystal formation of stable minerals, like the Pb biomineralization through fungi (Fomina and Gadd 2014, Rhee *et al*. 2014). Besides crystallization of soluble or insoluble organic or inorganic compounds, transport, uptake and intracellular sequestration plays an important detoxification role. Through this reaction with electrophilic xenobiotic compounds less toxic, water-soluble and extractable products are formed. (Field and Thurman 1996). The detoxification within the cell is devided in three phases: I oxidation, II conjugation of ion groups and III elimination of xenobiotic or metal complexes under ATP consumption. This elimination can takes place outside the cell, by the help of metal efflux transporter and followed enhanced efflux, intracellular, with glutathione chelation and subcellular, or vacuolar, by compartmentation of metal ion complexes, like for example the formation of glutathione-complexes (Bellion *et al*. 2006).

1.4.3. Glutathione S-transferase activity

Quantitatively a very potent natural antioxidant and instrument of cellular defense for eukaryotes is the detoxification enzyme gluthation S-transferase (GST). This enzyme

contributes a large superfamily of multifunctional proteins with a substantial role in cellular detoxification of exogenous and endogenous compounds (Frova 2006). Studies of glutathione (GSH) conjugations to hydrophobic molecules already started in 1959 (Barnes *et al*. 1959). Booth *et al*. (1961) identified the involved protein in transfer of thiol groups from the GSH tripeptide to the hydrophobic molecules.

Structurally the GSTs are ubiquitous tripeptides composed of three amino acids in the range [*N*-(*N*-L-glutamyl-L-cysteinyl)glycine]. It`s a principle innercellular detoxification phase II enzyme and catalyses the conjugation of tripeptide glutathione (GSH, -Glu-Cys-Gly), while cysteine is exchangeable by Ser, Tyr, Phe and Asn (Zhang *et al*. 2008). Non-polar compounds with an electrophilic centre; like carbon, nitrogen or sulfur atoms; can form exhaustive soluble, non-toxic peptide derivatives or metabolites for further excretion or compartmentation within phase III of the innercellular detoxification mechanism (Coleman 1997, Sheehan *et al*. 2001). While the specificity for compounds on this site is rather loose.

In the GST reaction (Fig. 5) the conjugation of reduced GSH to molecules with electrophilic centres happens to turn them in water-soluble compounds (Armstrong 1997, Hayes *et al*. 2005).

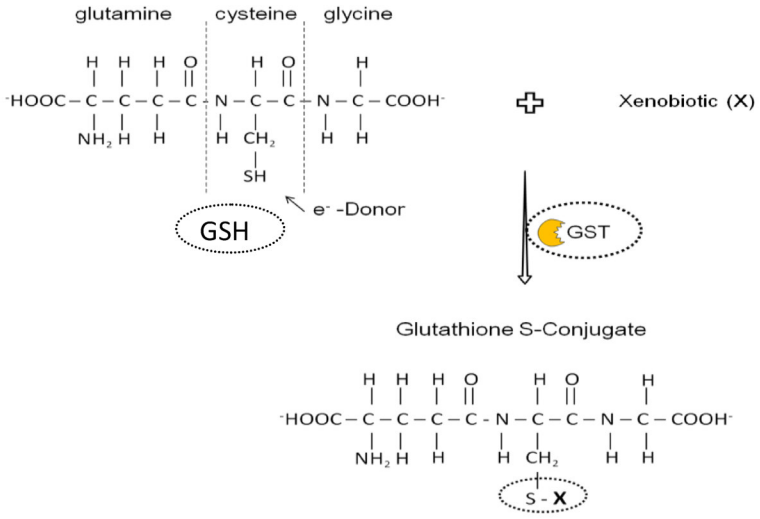

Fig. 5: **Glutathione S-transferase reaction**. The reduced GSH tripeptide reacts with the xenobiotic (X) under involvement of conjugation enzyme glutathione S-transferase (GST) forms a soluble and hydrophobic, less toxic and extractable glutathione S-conjugate with the thiol group of GSH (S-X).

GSTs are divided in different classes like cytosolic or soluble GSTs, microsomal or MAPEG GSTs, mitochondrial GST classified as kappa class and bacterial plasmid-encoded

fosfomycin-resistance GSTs (Frova 2006). An allocation of the fungal GSTs is discussed for omega class GSTs (GSTO) and xi-class next to the fungal GSTs, the GTTs. Fungal GSTs seems to be especially diverse in structure and function, caused by their substrate unspecificity. Therefore its generally problematic to categorize them into described classes after substrate specificity criterion. (McGoldrick 2005, Morel *et al*. 2009, Meux 2011). Fungal glutathione transferases (GTTs), GTT1 and GTT2, showed activity answering the commonly used GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Morel *et al*. 2009). Smirnova and Oktyabrsky (2005) described furthermore GSTs intracellular regulation of K^+ concentration, responses to temperature stress and GSTs positive role in osmoadaptation, similar to reactions induced by oxidative stress.

Next to the protection of cell destruction, GSTs are known to prevent fungal cells of metals and antifungal compounds (Veal *et al*. 2002). Admis *et al*. (2004) described GTT2`s (ScGTT2) involvement in Cd detoxification by catalyzing the formation of glutathione Cd-conjugates. This large field of diverse binding ligands, which are mainly hydrophobic, requires GSTs important role in detoxification of xenobiotic and innercellur toxic compounds and as class II biotransformation proteins (Hayes *et al*. 2005).

Genome data analyses of Morel *et al*. (2009) resulted in higher numbers of GSTs with higher complexity in ascomycetes and basidiomycetes, like e.g. ECM fungus *Laccaria bicolor*. The dimeric GST enzyme monomers obtain two domains: one site, a thioredoxin N-terminal domain, with conserved GSH binding G-site and the more variable C-terminal α-helical domain with GSH acceptor H-site (Armstrong 1997, Mannervik 2012). The soluble homo- or heterodimeric cytosolic GSTs with rather conserved N-terminal domains consists of βαβαββα topology for GSH binding and a variable C-terminal domain too (Oakley 2005).

1.4.4. Ectomycorrhizal fungi as biological filter

Extramatrical hyphae and mycelial cords do not only uptake nutrients and water. They do also vary in regard to inclusion of potential toxins and elements depending on the fungal species, hyphal growth in soil and host plant (Bowen 1994). All substances that potentially enter the plant root are channeled through the fungal mycelium based on the formation of fungal tissue around plant roots also known as short roots (Fig. 2). ECM fungi can protect plant cells from toxicity by interfering with the uptake of elements (Tamponnet *et al*. 2008), by metal efflux in tolerant ecotypes and by preventing metal overload of fungal and plant organs (Colpeart *et al*. 2011). All these effects contribute to phytostabilisation and the

preservation of intact ecosystems. Therewith alternative rhizoremediation strategies are natural attenuation, bioaugmentation and phytoremediation (Kuiper *et al*. 2003).

This allows the option to scavenge entering material already at the plant-fungus interface. Ectomycorrhizal fungi show different sensitivities to pesticides, especially fungicides. Laatikainen and Heinonen-Tanski (2002) proved effects ranging from massive growth inhibition up to toleration or even growth stimulation. Glyphosate, for example, a common broad-spectrum systemic herbicide, causes slight stimulations of ectomycorrhizae *Suillus* spec. (Laatikainen and Heinonen-Tanski 2002). Pesticides generally sprinkle the soil surface and the organisms therein. Due to the encasement of plant root organs by fungal mycelia, direct contact with the pesticides can be avoided. In this way pesticides may be filtered. Ultimately the fungal tissue is able to include (Donnelly *et al*. 1993), degrade (Meharg and Cairney 2000) and immobilize (Donnelly and Fletcher 1994) substances like pesticides.

For potential bioremediation approaches the accumulation of heavy metals into the cell wall of the hyphal mantle of ECM, *Pisolithus tinctorius* – oak, plays another important role (Gherghel and Krause 2012). Increased heavy metal concentrations in extramatrical hyphae assumes the filter option of the ECM. Blaudez *et al*. (2000) for example proved this for Cd. And Wilkins (1991) already showed that fungal symbionts like *Amanita* spec., *Paxillus* spec. and *Pisolithus* spec. have increased metal concentrations compared to the mycorrhizal plant symbionts birch, pine and spruce.

In general the tolerance to metals can be defined as capacity of organisms to exist within toxic substrates and their respective interaction of the genotype with its environment (Macnair *et al*. 2000). Based on filter effects used for tree establishment and development in toxic substrate, significant growth support is known of the early colonizer and generalist ECM fungus *Paxillus involutus* and the late colonizer, *Picea*-specialist *Tricholoma vaccinum* in co-cultures with *Pinus sylvestris* and *Picea abies*. The increase of biomass production of young tree seedlings was observed in axenic treatments with 10 mM CsCl directly after the inoculation of ECM fungi by Bizo *et al*. (2013).

Basidiomycetes are also known as efficient Cs accumulators (Dighton and Horrill 1988, Olsen *et al*. 1990, Haselwandter and Berreck 1994). ^{137}Cs was detected in fungal fruiting bodies along with analogous alkali metals like K^+, which where competitively taken up by fungal mycelia (Terada *et al*. 1998, Sugiyama *et al*. 2008). It is described that the uptake of radionuclides tends to follow the same pathways like analogous nutrients (Casadesus *et al*. 2008). Cesium is transported by the mobilization and uptake of K^+, by the

alkaline transporter, therefore K-related pathways in form of K channels are involved in the accumulation of Cs^+ (Schaller *et al*. 1990, White *et al*. 2003). Potassium channels are highly selecting monovalent cations in the range: $K^+ > Rb^+ > Cs^+$, whereby their selectivity for Na^+ is significantly lower (Doyle *et al*. 1998). Sr^{2+} is analogous transported to Ca^{2+}. It is known that Cs^+ and Sr^{2+} are accumulated in different parts of fungal fruiting bodies, e.g. lamellae and hat flesh, and that there are different accumulation preferences for elements in mushroom parts (Seeger *et al*. 1982).

In fungal communities a decrease of Cd and Pb in correlation with the P consumption is discussed by Jumpponen and Jones (2010). It is conceivable that polyphosphate granules can act as counterion in the vacuole and detoxify metals by removing from the cytoplasm (Kothe *et al*. 2002). The intracellular mechanisms include binding to organic acids, sulphur groups, (poly)phosphates and peptides, or the transfer into intracellular compartments like vacuoles and accumulation of reactive-oxygen species (Bellion *et al*. 2006).

1.5. Guttation in basidiomycetes and aquaporins

Guttation of liquid organic, cytoplasmic originated material, is mostly known from plants, but also fungi are able to exude droplets directly from mycelia (Dörfelt and Ruske 2014). The option to exude cytosolic water by the procedure of guttation can prevent bursting of the cells. In contrary to plants, which guttate by the help of special organelles called hydathodes (Taiz *et al.* 2010), fungi exude in form of guttation droplets (GD) over their hyphae (Weiler and Nover 2008).

The hyphal cell wall along the hyphal strands consists of a mixture out of (1-3)-beta-glucan together with amorphous chitin. At the hyphal tip the (1-6)-beta-glucan compartment is absent in contrary to the rest of the cell wall (Wessels 1999). The turgor pressure can act here as driving force in the wall expansion (Wessels 1999) and also for elongation of the hypha along with vesicle transport (Deacon 2006). This vesicle transport out of hyphae is discussed to take place mainly on hyphal tips, at the Spitzenkörper. There hyphal branches additionally split to form the apical vesicle cluster with a collection of vesicles at the hyphal tip. (Riquelme and Sanchez-Leon 2014, Riquelme *et al*. 2014). These options can give the cell wall on the very tip of the fungal hypha advantages to exudates (Fig. 6) and therefore exclude substances out of the cytosolic inner part to the extracurricular hyphal environment (Read 2011).

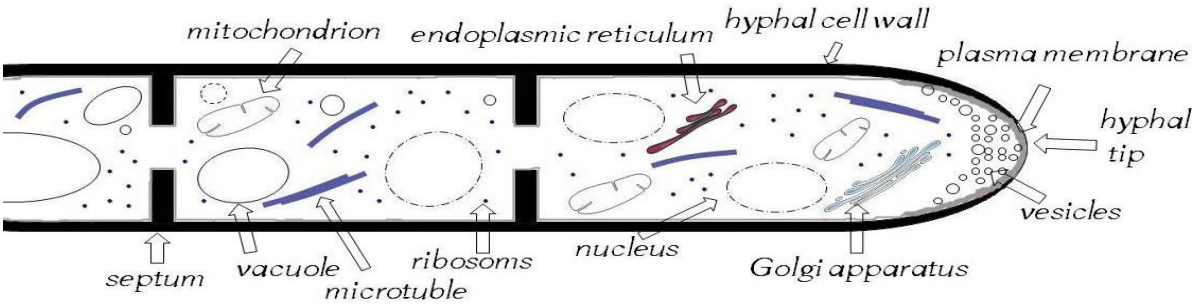

Fig. 6: **Scheme of the hyphal ultrastructure**. A septate hyphae with thinner hyphal tip, the Spitzenkörper, and cell organelles (modified after Deacon 2006).

The guttation process, is an active excretion of cell (cytoplasmic) water and dissolved material (Weiler and Nover 2008). It is described as phenomenon of fungal mycelia too (Sprecher 1958, Georgiou *et al*. 2006, Hutwimmer *et al*. 2010, Dörfelt and Ruske 2014). Exudation in droplets ocurred on media with different carbon sources (Hutwimmer *et al*. 2010). Remsburg (1940) and Buller (1958) already reported the formation of liquid droplets of fungi for basidiomycota. Sprecher (1958) declaired more detailed, that the exuded droplets are produced on aerial parts of the mycelia. Such droplets can consist enzymes, solutes (Colotelo *et al*. 1971; Colotelo 1973, 1978) and toxins (Grovel *et al*. 2003, Gareis and Gareis 2007). The key question to fungal metamorphosis lies in understanding how that, which is outside a hypha can influence that which goes on inside (Georgiou *et al*. 2006) and the other way around. After Deacon (2006) is the exudation of cytoplasmic cell material physiological possible. Exocytic events are described while vesicles docking and fusing at the furrow area of the cell wall (Militello and Colombo 2013). But clear scientific evidence on function of fungal droplets and it's ecological role is lacking. Jennings (1991) saw GD as sign for unfavorable water potentials. Guttation droplets else function as reservoir for metabolic by-products (Sun *et al*. 1999, Pereira *et al*. 2012), reserves, secondary metabolites and enzymes (Mcphee and Colotelo 1977, Colotelo 1978).

As well the membrane integrated selective water transmembrane proteins: aquaporins (AQPs) are targets for investigations on the occurrence of GD. AQPs are membrane integrated proteinaceous pores, which enable water transport by providing membranes at low temperatures several times more permeable (Borgnia *et al*.1999). Two types of AQP are characterized. AQP - for selective water transport, which are bipermeable for H_2O molecules and aquaglyceroporines - with extended transport channels, which can pass next to water glycerol and other small molecules.

The AQP superfamily, consisting of hydrophobic proteins with six membrane-

spanning α-helixes, with a molecular weight of 26–34 kDa and a water flux range in two ways (Verkman 2005, Fig. 7).

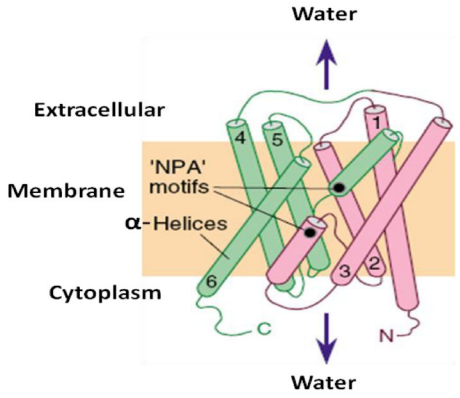

Fig. 7: **Scheme of crystal structure of a AQP monomer and its tetrameric assembly in membranes**. Transmembrane 1-6 α-helical domains surrounding a water pore with indicated conserved `NPA`motifs. (slightly modified after Verkman 2005).

The cellular water permeability within solutes faces three impediments: the cell wall permeability, the diffusion of lipid bilayer and proteinogenic pore. The major intrinsic proteins (MIPs) accommodate a wide range of substances. They conduce the transport of: water by affecting the membrane permeability (AQP), uncharged neutral molecules and solutes, e.g. glycerol and ammonia (aquaglyceroporin), or both over the plasma membrane and cell wall barrier. As well they are discovered in all kingdoms from archea and eubacteria to fungi, animals and plants (Engel and Stahlberg 2002). AQPs are structured by transmembrane helices forming hourglass like pores (Jung *et al*. 1994). A central channel is separately crossed by water and solute molecules (Gena *et al*. 2011).

Whereby the multifunctional AQPs transfer not only water but also gas and solute permeabilities (Nehls and Dietz 2014). Silver, as known blocking element for thiol-groups, can inhibit transport *via* AQPs (Nagarajan and Ebbs 2007). Free silver ions act strongly fungicidal at low doses, which finds wide use in the clothing industry (Ratte 1999, Morones *et al*. 2005).

1.6. Mesocosm experiment and natural succession

Mesocosm experiments are advantageous research tools to control and stabilize growth conditions by focussing soil ecological processes (Hantschel *et al*. 1994). They can

show miniature ecosystems in defined scales and their diversity and improving variation in species composition (Dai *et al*. 2009). Changes in soil structure are easily detected on this evidence under controlled biotic and abiotic conditions. Experiences from Yamada *et al*. (2007) with different systems showed more success of experimental set-up from lasting pot cultures. Another big advantage of mesocosm experiments, is the option to focus especially on the distribution and content of organo-mineral associations in small scales (Khasa *et al*. 2001). The option to use defined soil amounts to quantify element turnover, for studies of element transport, makes mesocosm systems very eligible (Tuovinen *et al*. 2015).

The construction of the mesocosms can be diverse in size and construction. Several authors, e.g. Blechschmidt *et al*. (1999), Dai *et al*. (2009) and Tuovinen *et al*. (2015); described experiences with mesocosm system sizes with amounts of 8.0 kg - 25 l volume, around 23 - 30 cm height and 14 - 23 cm diameters.

The mesocosm substrate in this study origins from a former uranium mining leaching heap, the territory of Ronneburg, Thuringia, Germany (Gauß-Krüger coordinates: northing 4511140, easting 5632824). Between the years 1946 and 1990 around 113000 t U were mined in this area (Wismut GmbH 2001, 2005). Mine dumps are anthropologic developed and constisting of natural substrates. But the natural physico-chemical characteristics are changed by strong mechanical load from disposal and aggregation of solum material and quarterian sediments of the substrate layers. (Beckers 2005). In the territory of Ronneburg U layers from the upper Ordovicium and lower Devonian are present. The primary source of U, the black slate, is rich of organic carbon (5-9%), sulfide (2-3.5%), U (30-60 ppm) and other trace elements. Decomposition resulted in dislocation of U from the oxidation zone in the reduction zone of the substrate. (Lange and Freyhoff 1991). Besides high U contents, sulphidic conjunctions with Pb, Zn, Co and Ar are representative in these layers (Gartzweiler *et al*. 1997).

In the Eastern Thuringian post mining landscape, heavy metals and radioactive Ra are spread *via* the atmosphere or the water path. As consequence of the dust formation heavy metal loaded particles can be spread large-area and deposit on substrates. Through appliance of acid mine drainage (AMD) heavy metals can arise and resolve. On this way mining substances can reach and become transported by the phreatic water. (Beckers 2005). Carbonate rich substrate with neutral pH conditions covers the origin substrate. This boulder clay, the sandy loess loam, originates from the sand pit Starkenberg, Saxony, Germany. (WISMUT GmbH 2010, personal communication with M. Köhler). Both substrates were used for mesocosm experiments.

Under influence of an establishment of ECM formation, with its associated improvements for the soil microstructure of the tree-fungus partnership (Marcowicz *et al*. 2015), the development of the structure of the soil substrate was performed. The main claim of the mesocosm experiment was focussed on soil parameters and the development of the plant-fungus diversity with included changes of element concentrations. A defined soil volume was chosen and focussed on the change of soil elements influenced by the ECM. Through the soil characteristics given conditions the plant cover adjusted with the heap originated diversity of plants. The insisted individuals are mainly the pioneer and less demanding plants *Pinus sylvestris* and *Picea abies* together with their fungal partner and extensive heavy metal accumulating basidiomycetes *Paxillus involutus, Pisolithus tinctorius* and *Tricholoma vaccinum*. Thereby more detailed information about the providing function of mycorrhiza fungi in soil decontamination and soil renaturation by the help of detoxification abilities of ECM should become highlighted. And moreover besides the sequestration, the move of certain elements should become studied.

1.7. Aim of the study

The aim of the study was to identify mechanisms of sequestration of heavy metals and radionuclides and the process related to tolerance. The fungal impact was studied in and around the ECM system of the early colonizer fungi *Pisolithus tinctorius* and *Paxillus involutus* and the late colonizer *Tricholoma vaccinum* in symbiosis with the tree partners *Pinus sylvestris* and *Picea abies*. A meaningful and in remediation questions applicable function of the ECM is its effect as biological filter. ECM plays a crucial role in mobilization and immobilization of xenobiotics in general, especially metals and radionuclides. Here, the intracellular immobilization by the glutathione S-transferase and extracellular metal tolerance through guttation were focused upon. The following hypotheses are to be tested:

1. Natural ECM diversity is influenced by the mobile metal concentration of the soil.
2. Morphotyping of the ECM community in metal contaminated sites is correlated with analyzes of determined elements and supports the knowledge to understand sequestration abilities of the ECM. The occurrence of the ECM fungi *Pisolithus*

tinctorius, *Paxillus involutus* and *Cenococcum geophilum* can therefore be applicable for remediation, demonstrated in a mesocosm experiment.

3. The development of ECM communities can be modified by inoculation.
4. The ECM diversity can change mobile metal concentrations in soil.
5. ECM supports the removal of the elements Cs, Pb, U, Sr, Ni and Cd from the bioavailable and soluble soil fraction of the soil metal pool.
6. Fungal metal tolerance involves GST activity.
7. Exudation of metals by fungi is possible with guttation droplets.
8. Guttation can be understood as process of attracting the fungal environment.

2. Material and Methods

2.1. Organisms, media and chemicals

All in this study used plant and fungal species are listed in Tab. 1. They originate from the JMRC (Jena Microbial Research Centre of the Friedrich Schiller University Jena, Germany).

Tab. 1. Used fungi and plant species.

Species	Strain	Origin	Life strategy
Paxillus involutus Brown roll-rim	RK081020	Oak, Ronneburg, Thuringia, Germany, collected by S. Formann	Non-specific ECM
Pisolithus tinctorius Bohemian truffle	FSU: 10019	Kauern, test field Gessenwiese nearby Ronneburg, Thurinia, Germany, collected by M. Gube, JMRC 51°51`19.92°N 12°8`53.66°E	Non-specific ECM
Tricholoma terreum	MG091108_03	Pine, Göttingen, Kerstlingeroder field, collected by M. Gube 51°31`21.42°N 10°0`11.80°E	Specific ECM with pine
Tricholoma vaccinum Scaly knight or fuzztop	GK6514	Spruce, Nassereit, Austria, collected by G. Kost	Specific ECM with spruce
Schizophyllum commune	FSU: 3214 x FSU: 2896	Mooibroek *et al*. 1990, JMRC	Saprophyte
Schizophyllum commune	Δ*Ku8070* T 14(4) x C6	de Jong *et al*. 2010	ΔKu mutant, transformant with *ura1* and *Ku8070* double deletion (stress sensitive; Madhavan 2014), saprophyte
Picea abies (L.) Karsten Norway spruce		Thuringian forestry office Schmalkalden, Germany	Forest tree
Pinus sylvestris (L.) Scots pine		Thuringian forestry office Schmalkalden, Germany	Forest tree

All used additives and media components of this work were purchased from the German companies Carl Roth GmbH Karlsruhe, Merck Darmstadt and Sigma Aldrich Steinheim.

All culture media (Tab. 2) are sterilized 30 minutes at 121 °C and 1 bar. For fungal cultures the medium was poured in 94 x 15 mm petri dishes, for tree–fungus sandwich–

cultures in 145 x 20 mm petri dishes and for germination of the tree seeds 250 ml of the germination agar was poured in 500 ml Erlenmeyer flasks. Cellophane sheets had been used for ECM fungal mycelium and sandwich culture plates. Therefore cellophane sheets were cut into 85 mm wide circles, boiled in distilled water to remove any production residue and the absorption of water quantities with final autoclaving. Afterwards each culture plate was covered with cellophane sheet before inoculation.

Dishes were sealed with Parafilm to avoid contaminations and stored in climate chambers (KBWF-720 L, Binder, Germany), with 12 hour day-night cycle and temperature alteration of 23 ° C / day to 17 ° C/ night at 80% relative humidity.

For tree seedling germination seeds were rubbed to remove the seed detached wax layers and subsequently swelled in water overnight. At the following day, the surface sterilization of the seeds was performed with shaking for 1-1.5 h in 30% H_2O_2. Afterwards the seeds were rinsed 3 times in *Aqua dest*. using sterile conditions at the clean bench. Subsequently 15 seeds were transferred onto each germination medium using sterile spatula or tweezers. After 2-6 weeks, after occurrence of secondary roots, the seedlings were separately transferred onto MMNa medium with addition of fungal mycelium.

Tab. 2: Growth media of fungal and tree cultures.

Fungal growth medium	**Content**	**Organism**	**Culture tubes**
MMNb ½	after Melin 1921, Norkrans 1949, Kottke *et al*. 1987, with glucose, with 4.9 mM ≙ 1 g L-tryptophan	*P. tinctorius*, *P. involutus*, *T. vaccinum* *S. commune* ΔKu8070 T 14(4) x C6	85 mm petri dishes
Sandwich culture			
MMNa Trace element solution	after Melin 1921, Norkrans 1949, Kottke *et al*. 1987, without glucose after Fortin and Piche 1979	*P. abies*, *P. sylvestris*, *P.tinctorius*, *P. involutus, T. vaccinum*	135 mm petri dishes
Germination cultures			
Germination medium	after Chilvers *et al*. 1986, with piperazine	*P. abies*, *P. sylvestris*	500 ml Erlenmeyer flasks

All used chemicals and antibiotics in this work were purchased from the German companies Carl Roth GmbH Karlsruhe, Merck Darmstadt and Sigma Aldrich Steinheim.

Solutions of different salts (Tab. 3) were filtered for sterilization with Rotilabo® – syringe filters pore size 0.22 µm, Carl Roth GmbH, Karlsruhe, Germany. After cooling the autoclaved medium at 65-70°C the sterile filtered chemicals were added.

To measure and detect the glutathione S-transferases (GST) activity a commercial kit system, the Glutathione S-transferase (GST) Assay Kit from Sigma-Aldrich Co., Taufkirchen, Germany with its attendant chemicals (reduced L-glutathione, pure GST and 1-chloro-2,4-dinitrobenzene (CDNB)) was used.

Tab. 3: Metal concentrations of fungal and sandwich cultures, GST and guttation experiments per L.

Growth of fungal and sandwich cultures	
Metal salt concentrations	0.1 / 0.4 / 0.5 mM $CdCl_2$
	5 / 10 / 15 mM CsCl
	1 mM $NiCl_2$
	0.5 / 1 mM $PbCl_2$
	40 mM $SrCl_2$
GST and guttation experiments	
Fungicide	0.002 mM azoxystrobin/strobilurin
AQP blocking reagent	0.01 mM acetazolamide (AZA)
	0.01 mM AgCl
	0.01 mM Ag NO_3
PBS buffer 1x (pH 7.4)	137 mM NaCl
	10 mM Na_2HPO_4 * 2 H_2O
	2.7 mM KCl
	2 mM KH_2PO_4
Thin layer chromatography	
Sugar detection	Acetonitrile
Amino acid detection	1-butanol
	glacial acetic acid
	A. dest.
Reference	10 mg / ml D-ribose
	10 mg / ml L-glutamine
Sequential extraction	
Fraction I	1 M NH_4 NO_3
Fraction II	1 M NH_4 OAc
ICP-MS/OES	
Element digestion	concentrated HNO_3

2.2. Characterization of ectomycorrhiza

The morphological determination of ECM short roots are performed after R. Agerer`s short description of the "Colour atlas of ectomycorrhizae" (2012). Additionally sequence analyses of internal transcribed spacer (ITS) data of short roots were performed (White *et al*. 1990, Gardes *et al*. 1991). Therefore DNA of short root samples was extracted with Power Soil Kit (MO BIO) and if necessary the DNA stored at -20°C. For control gel electrophoresis of the received DNA with blue marker and λ *Pst*I weight marker in 0.8% agarose gels was performed.

For amplification of ITS fragments polymerase chain reaction (PCR) was performed. with a thermocycler of Biometra GmbH (Göttingen, Germany) using specific reagents (Tab. 4).

Tab. 4: Used reagents for amplification of DNA-fragments by PCR.

ITS-PCR reagents	**Concentration**	**Amount**
Taq-Polymerase		0.25 µl
dNTP`s	2 mM	1 µl
Primer ITS1 (TCCGTAGGTGAACCTGCGG)	10 mM	5 µl
Primer ITS4 (TCCTCCGCTTATTGATATGC)	10 mM	5 µl
Taq-Polymerase buffer		5 µl
DNA		1 µl
Aqua dest. Sterile	to fill up to 50 µl volume	

After PCR of the following conditions: 5 min. 95°C denaturation, 30 sec. 95°C, 30 sec. 56°C annealing, 50 sec. 72°C elongation, 10 min. 72°C; 39x gel electrophoresis of the products was performed within a 1.8% agarose gel using blue marker and λ *Pst*I-size marker. After the staining in 0.1 µl/ml ethidium bromide solution, DNA was visible under UV light. Under these conditions DNA bands were cut out of the agarose gel and purified with Jetsorb DNA purification kit (GenoMed GmbH, Leinefelden, Germany).

The sequencing of DNA fragments was done by the company JenaGen (Jena, Germany) after Sanger *et al*. (1977). The software Chromas, DNA Star, Bioedit, MAFFT v7 was used to edit and align sequences.

The sequences were subsequently matched by the BLAST algorithm in the NCBI gene data base. Resulting alignments were generated with the first best hits.

2.3. Concentration of elements in ectomycorrhiza

Several fungal fruiting bodies were collected in different seasons from July until November 2009/2010 in the area of the former uranium mining heap Ronneburg, Thuringia, Germany. For metal concentrations fruiting bodies, total extraction and ICP-MS/OES was applied (Heinrichs and Herrmann 1990). Fungal material was dried at 60°C until weight constancy, subsequently mortared with liquid nitrogen and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) for trace elements or inductively coupled plasma-atomic optical emission spectrometry (ICP-OES) for alkali and alkaline earth elements. Advantages of the ICP techniques concede the detections of elements in low amount ranges from pg on.

For the ICP-MS measurements (XSeries II, Thermo Fisher) or ICP-OES (725 ES, Varian) an amount of 100 mg powdered fungal tissue was acidified with concentrated HNO_3 and prepared by micro wave digestion. Resulting elemental concentrations were calculated for the respective BCFs, to exemplify elemental uptake of fungi.

2.4. Mesocosm experiments

The used soil was kindly provided by the WISMUT GmbH. In the mesocosm experiment used substrates originate from a former uranium mining heap nearby Ronneburg, Thuringia, Germany with the Gauß-Krüger coordinates: 45°11′140″E and 56°32′824″N. In total ten 30 L volume pots of food-safe polyvinyl chloride (PVC) with heap material (compacted, clayey, iron rich, ferric red colored; n=7) and loess loam (carbonate containing; n=3) were analyzed for over a time period of 18 months under natural conditions (Fig. 8).

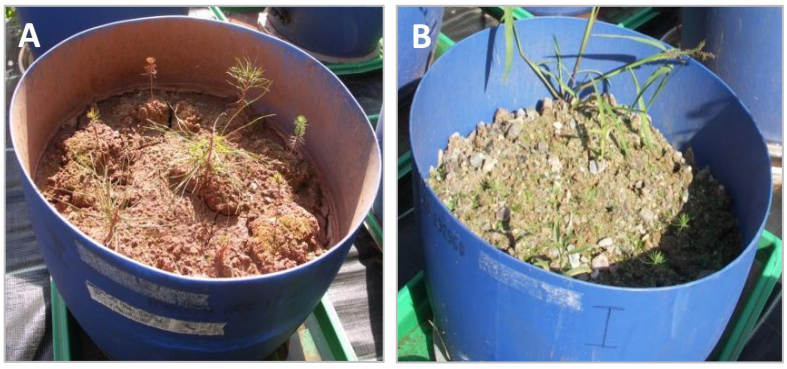

Fig. 8: **Mesocosm substrate**. Substrate of the former uranium mining heap Ronneburg, Germany in 30 L pots. **A**: Heap material, compacted clayey iron rich substrate. **B**: Applied material, sandy loess loam.

Seedlings of fast growing pioneer wood *Pinus sylvestris* and *Picea abies* were pre-germinated in smaller planters. Eight to nine young trees of each were transferred into the mesocosms after four to six months. From axenic cultures 0.3-0.4 g fresh mycelium of the basidiomycetes *Pisolithus tinctorius, Paxillus involutus* and *Tricholoma vaccinum* were inoculated immediately in addition. The fungi *P. tinctorius*, *P. involutus* and *T. vaccinum* were pregrown as pure culture on MMNb ½ medium before inoculated into the pots. Four pots of heap material and 2 pots of sandy loess loam were inoculated with ECM fungi.

To quantify the elemental content of the soil substrate at the starting point and after the experimental period, sequential extraction mobile and easily mobilizable fractions 1 and 2 (Zeien and Brümmer 1989) with subsequent ICP-MS/OES was used. Statistical analysis of element contents was performed after testing normal distribution using t-test. For unequal variances the Welch-test and for analysis of variances the U-test (Mann and Whitney). For single factor variance analysis the H-test (Kruskal-Wallis-test). All tests were performed using R *via* RStudio Inc. software. A determination of minerals occurring on the substrate, after storage protected from rain and wind, was done. After collection of the visible minerals X-ray diffraction (XRD) was performed.

2.5. Glutathione S-transferase activity

To measure and detect glutathione S-transferase (GST) activity, a commercial kit GST-Assay (Sigma-Aldrich, Steinheim, Germany) was used. This based on the reaction:

$$\textbf{(2)} \qquad \textbf{GSH reduced (G-SH) + CDNB} \xrightarrow{\textbf{GST}} \textbf{GS-DNB-Conjugate + HCl}$$

with the GST-substrate CDNB (1-Chloro-2,4-dinitrobenzol) kinetically at 340 nm and 1 U unit of GST activity is defined as 1 mmol GS-DNB formed per minute. To test GST activity directly in ECM, sandwich-cultures were cultivated for 3 months and mycelium and seedlings ground in liquid N_2 with crown and stem separate from the roots with mycelium (Fig. 9). For control axenically grown fungal mycelium was used. 100 mg were transferred into 15 ml Falcon tubes with 1ml PBS buffer (Tab. 3), vortexed and centrifuged for 3 min. at 4°C with 14.000 rpm. GST activity was measured in 96-well plates (Sigma-Aldrich, Steinheim, Germany) in a VERSAmax microplate reader (Molecular Devices, Sunnyvale, California, USA). After 2 min. lag time the absorbance was measured at 340 nm every 30 seconds for 10 minutes in 3 replicates. Well plates and additives had been kept on ice the whole procedure long.

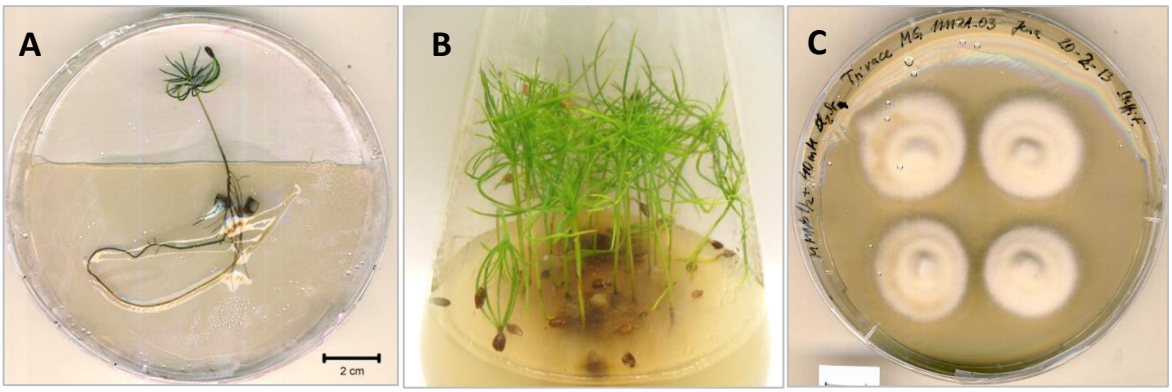

Fig. 9: **Culture sytems A** Sandwich culture of ECM partners *P. abies*, *P. involutus* and *T. vaccinum* on MMNa medium after 3 months. **B** Tree seedlings of ECM *P. sylvestris* together with *P. involutus* on germination agar after 3 months. **C** ECM fungus *T. vaccinum* as single fungi culture on MMNb ½ with 40 mM $SrCl_2$ after 2 months.

The rate of color development over blank was used in the following equation:

$$\frac{(A_{340})/\text{min.} \times V\,(\text{ml})}{\varepsilon_{mM} \times V_{enz}(\text{ml})} = \mu\text{mol/ml/min}. \quad (3)$$

with extinction coefficient for CDNB ε_{mM} = 5.3 $mM^{-1}cm^{-1}$, reaction volume V = 0.02 ml and volume of the tested sample V_{enz} = 0.02 ml. The results were analysed using IBM SPSS (Statistics Version 22) after a paired t-test for stem versus roots. Metal treatments and different species were analyzed by one-way ANOVA. The Kolmogorov-Smirnov test and the Levenes tests were performed, to confirm the data met the assumptions of one-way ANOVA. If the data did not meet the assumptions, the non-parametric Kruskal-Wallis test was used. If significant differences were observed, a Mann-Whitney U test was used to compare combinations.

2.6. Guttation

Fungal mycelium was cultivated on MMNb ½ medium (Tab. 2) under axenic conditions. After 4-12 weeks GD were collected using 0.1 ml pipette tips or 5 µl micro pipettes with ring marks (Hirschmann, Germany). The liquid was collected in borosilicate glass (Rotilabo, Germany) and stored at 4°C. The stereomicroscope Stemi 2000-C and Axioplan 2 (Carl Zeiss, Germany) were used for documentation with D10ZNC software program Spot 4 (Visitron, Germany). A volume of 50 µl of GD was transferred in 1.5 ml reaction vessel and subsequently centrifuged for 10 min. at lowest speed in vacuum

centrifuge, Savant SpeedVac. The residue was dissolved in 10 µl methanol and punctiform added to silica gel foils, $60F_{254}$ (Merck, Darmstadt, Germany).

The presence of D-ribose and L-glutamine was measured by thin layer chromatography (Kraus *et al.* 1996) with UV detection. Using 10 µl diluted D-ribose or L-glutamine for reference. After the run (acetonitrile:*A*. *dest*. = 85:15 for sugars and 1-butanol:glacial acetic acid:*A*. *dest*. = 40:10:10 for amino acids), dots were tagged under visible and UV light. Sugars were stained after vaporization for some minutes with ammoniac and heating for 1 min. at 100 °C. Yellow coloring occurred by this procedure for sugars.

Amino acids were analyzed by spraying with ninhydrin reagent (0.1 g ninhydrin in 50 ml ethanol) followed by heating for 1 min at 100 °C resulting in blue or purple stained amino acids.

Elemental contents in guttation droplets were analyzed by ICP-MS/ ICP-OES (725 ES, Varian) after acidification with concentrated HNO_3 and micro wave digestion.

3. Results

3.1. Ectomycorrhiza diversity at the former uranium mining site

Short roots of the ascomycete *Cenococcum geophilum* were identified in the soil pots, see Tab. 5 and Fig. 10 with respective characteristics:

Tab. 5: Determination of short roots after R. Agerer, 2012 "Colour atlas of ectomycorrhizae".

Quality of short root	Characteristic
Type of ramification:	simple, unramified
Length of the ECM system:	up to 3 mm
a) Length of unramified ends:	2 mm
b) diameter of unramified ends:	up to 298 µm
c) diameter of axis:	ca. 298 µm
Shape of unramified ends:	straight, unbent
Distinct features of mantle-surface:	grainy, long spiny-hyphae
Rhizomorphs:	None
Emanating hyphae:	No
Colour of ectomycorrhiza:	Black
Quantity of ECM per root length:	17/cm
Result:	*Cenococcum geophilum*

Fig. 10: **ECM short roots of *C. geophilum* with *P. sylvestris***.

Sample sequence was identified with BLASTN algorithm as *Cenococcum geophilum* (see alignment Fig. 11).

Fig. 11: **Alignment of *C. geophilum* fragment compared to data bank sequences of *C. geophilum*** (FJ378841.1, DQ474370.1, KC702626.1). Similarities are dark underlined.

3.2. Concentration of elements in ectomycorrhiza

Contents of the elements Cd, Cs, Sr, Pb, Ni and U from fruiting bodies (Tab. 6) were analyzed by Sequential extraction method and declared by BCF values.

Tab. 6: Bioconcentration factors (BCF) of two different fungal fruit bodies and elemental quantity in µg/g from the heap site Ronneburg, Thuringia, Germany (data acquisition in 2009).

Species	Cd	Cs	Ni	Pb	Sr	U
Pisolithus tinctorius (BCF)	7	**12**	9	**40**	**11**	**28**
Paxillus involutus (BCF)	2	**66**	3	2	9	5
Soil bioavail. (µg/g)	0.009	0.11	2.1	0.12	0.83	0.24
Soil total (µg/g)	0.37	3.33	23.8	29.4	131.4	7

Both ECM fungi *Pisolithus tinctorius* and *Paxillus involutus* showed clearly an uptake of all investigated elements Cd, Cs, Ni, Pb, Sr and U, but especially high accumulation factors for radionuclides, indicated by BCF > 1 (Tab. 6). While *P. tinctorius* showed tendencies to prefer accumulation of Cs, Sr and U in high ranges, very high accumulation factors were observde for Pb. *P. involutus* showed accumulation of Sr, U and especially very high BCF values of Cs.

3.3. Uptake of metals in ectomycorrhiza

3.3.1. Substrate determines metal uptake

The used heap material was situated under the pit, enriched with mining residual additives and after finishing mining activities used for pit filling. The area of the heap passed more than 40 years of mining activity, with acid mine drainage, leaching and soil movement. This material consists of peripheral upcoming, initial filling material of the former uranium heap and is composed of overburden caused by the mining activities. The heap material, a quarterian ferric material, can be characterized dense, compacted, loamy, clay and iron rich with high skeletal proportion. The skeletal counterpart was represented especially by the uranium base: black slate. As a quaternary relict it consists of quaternary belt layers. The applied acid mine drainage resulted in contaminant influx as well as heavy metal and radionuclide accumulation. The soil substrate is therefore less developed with very slow organic entry. During the experimental processing an acidic pH of 3.2 in combination with heavy metal and radionuclide accumulation induced less restricted water flux with little

organic input. The tendency of silting up was a big problem over the experimental period, due to large amounts of very small soil particles.

To improve substrate properties the heap material was covered by lidding material loess loam. This loam is a sandy clayey and carbonate rich till with neutral pH around 6-7.5 (Fig. 8) originated from the sand pit Starkenberg, Saxonia, Germany (personal communication M. Köhler, WISMUT GmbH Ronneburg, Germany). It contains of high skeletal content with quartz, marl and limestone fragments and was primarily used as coverage and soil improvement. The physiologically more bearable pH of around 7 with a carbonate amount of around 7 mass% improved the biotic and abiotic soil structure and soil conditions. This induced enhanced plant growth and self colonization of herbs that partially concludes in the immobilization of elements. It was used as control material (n=3) in the mesocosm experiment (for detailed substrate characterization see 3.3.1.). These two substrates (Fig. 8) were investigated regarding to mineral and elemental contribution as well as soil living and promoting ECM colonization along with their development. Additionally the distribution of different prominent minerals and their specific proportion in the acidic heap material is depicted. The investigated cover substrate loess loam showed clear structural differences (Fig. 12).

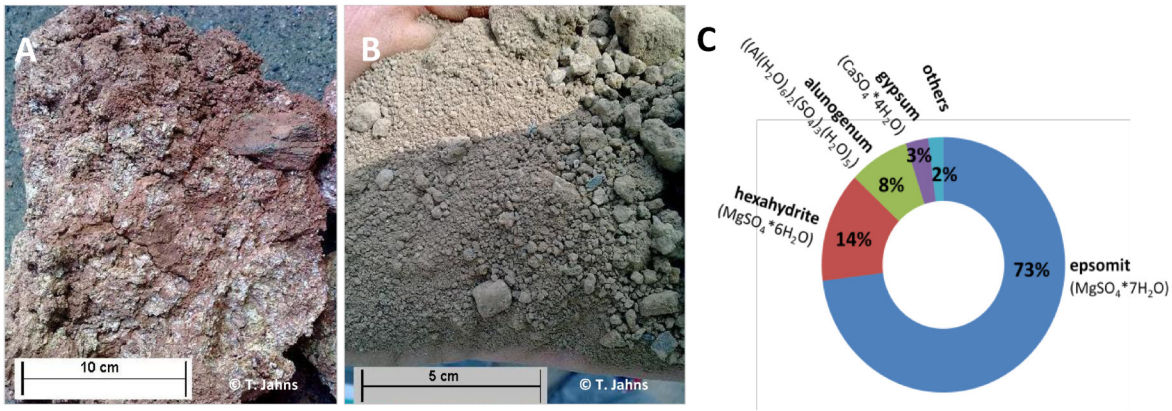

Fig. 12: **Substrate of mesocosms**. **A:** Dense clayic, loamy heap material. **B:** Sandy clayic loess loam. **C:** Distribution of different prominent minerals and their specific proportion in the acid mine drainage treated heap material at the time of experimental set up. (pictures by T. Jahns).

The oxidation of reduced S-compounds, as relict of the acid mine drainage treatment, was promoted by evaporation and low organic layer development on the substrate. High amounts of SO_4^{2-} in pore water induced crystal formation on mineral surfaces (Fig. 13). The

evaporation of less overgrown substrate resulted in crystal and crust formations consisting of SO_4-associatives: gypsum ($Ca(SO_4)*2H_2O$), hexahydrit ($Mg(SO_4)*6H_2O$) and epsomit ($Mg(SO_4)*7H_2O$) (Fig. 13). Mineral blooming could be found on the substrate in dry and calm periods.

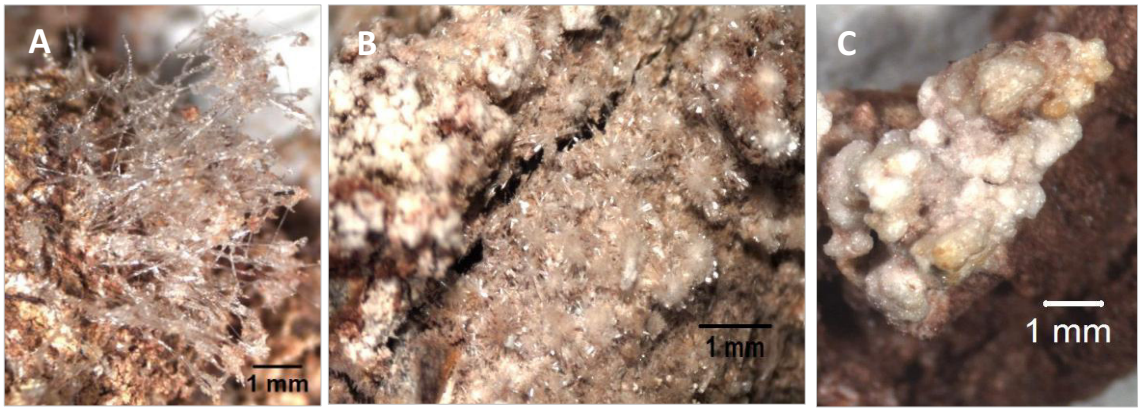

Fig. 13: **Mineral efflorescence and crystal formation at the mineral surface of heap material hexahydrit**e ($Mg(SO_4)*6H_2O$) **A**: fibrous aggregates, **B**: efflorescence and **C**: Epsomit ($Mg(SO_4)*7H_2O$) crusts.

3.3.2. Heap material

By comparing the two different soil types after 18 months the behavior of non-essential and essential elements in the soil structure chemically and physically showed clearly variations under influence of ECM and plants. Fig. 14 represents the distribution of non-essential elements Cs, Sr, U and essential elements Ca, K, Mg and S of the heap material. In comparison to the control samples without plants or inoculated fungi higher bioavailability of Cs with 40% and lesser bioavailability of Sr with > 20% appeared in the root space (3 cm soil depth) as well as in deeper, root indirectly influenced, soil (50 cm soil depth). In contrast, the presence of ECM and plants reduced the amount of Cs in root space and deeper soil regions, compared to the control samples without plants. Sr showed higher amounts with 40% in ECM and plant treatments whereas lowest amounts of 25% with low tendency to enrich in deeper soil zones in treatments without any plants.

While the availability of U mainly depends on its chemical state, it`s distribution seems to have higher amounts of 42% in the root space with ECM. In deeper zones U seems to be less available and showed indirect influence of ECM and plant roots, but generally restored or in some extent enriched in deeper soil zones (Fig. 14).

The essential element contribution in the heap material seems to be more balanced. Ca, Mg, S showed equal distribution with 20:30:35 and lesser availability of K 10%. But in

presence of ECM more Ca 30%, lesser Mg 25% and S 30% tend to be available. Whereby root space and deeper soil zones distribution are not clearly different in the heap material (Fig. 14).

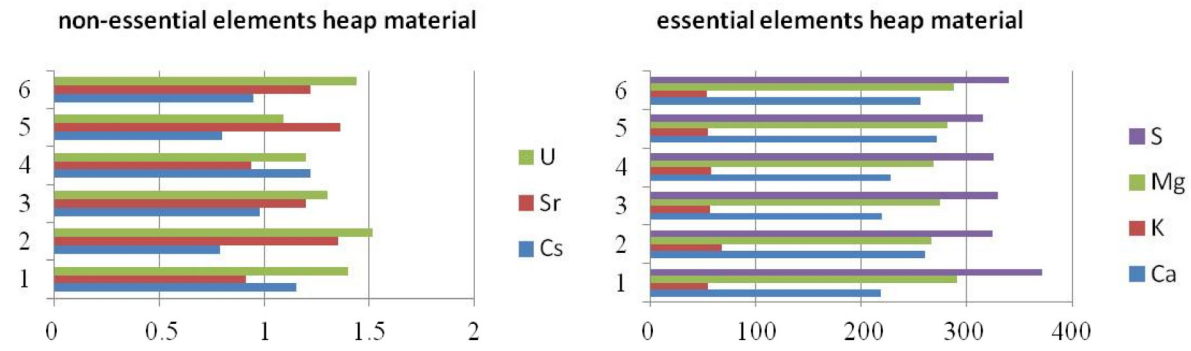

Fig. 14: **Content of elements in heap material after experimental treatment**. **1-3**: root space, 3 cm soil depth with **1** control without plants, **2** ECM plants, **3** plants without ECM. **4-6**: deep zone, 50 cm soil depth without roots with **4** control without plants, **5** ECM plants, **6** plants without ECM.

By the help of a logarithmic scaling of the proportions of non-essential elements of the heap material a general lesser amount of available elements can be seen under influence of ECM compared to less and non overgrown substrate (Fig. 15). A less pronounced effect but still the tendency of higher availability of elements with ECM can be determined by comparison of the essential elements (Fig. 15). This underlines that ECM is involved in the determination of non-essential elements and concurrently influences the availability of essential elements, not only for self-establishment of the tree-fungus symbiosis.

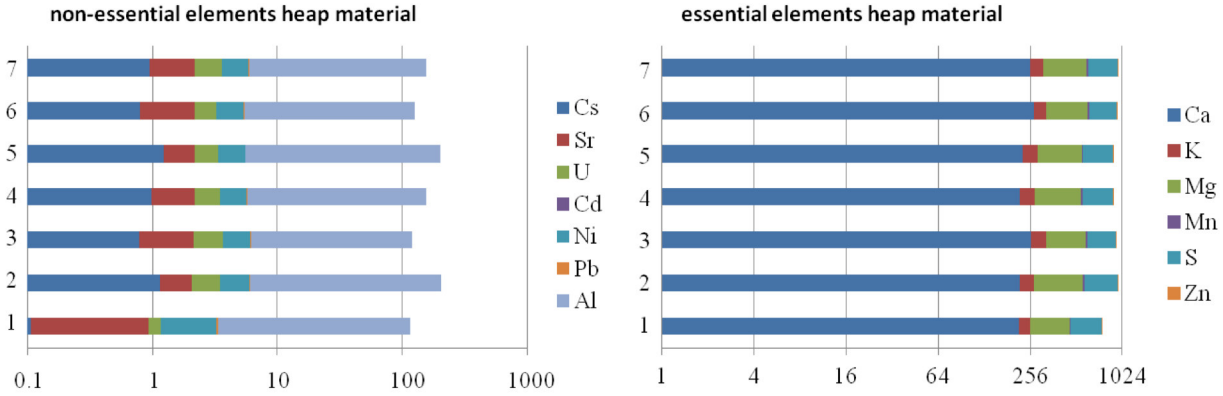

Fig. 15: **Logarithmic scaling of proportions of non-essential and essential elements of heap material after experimental treatment in total**. 1: original untreated soil, 2-4: root space 3 cm soil depth, 2: control without plants, 3: ECM plants, 4: plants without ECM, 5-7: deep zone 50 cm soil depth without roots with 5: control without plants, 6: ECM plants, 7: plants without ECM.

In conclusion the mobility of selected heavy metals and radionuclides in the heap substrate exemplifies the results of Sequential extraction method with following ICP-

MS/OES. To analyze elemental bioavailability the first two fractions of seven in total were focused. The mobile and the easy delivering fraction thereby being best bioavailable compared to the following six additional fractions (Fig. 16). The results show, that among others the elements Cd, Cs, Sr, and Ni are most mobile in the first fraction. Furthermore, next to the non-essential and essential elements like Mn and S (Fig. 16), appeared an increased mobility as well as higher availability in the first fraction. Compared to the first no different mobility of As, Fe and P can be seen in the second fraction. The U in the second fraction clearly showed higher mobility. Therefore it is not directly bioavailable in the rhizosphere, but could be available through the influence of root exudates from the later deliverable fractions. This means that the uranium mobility may depend on longer and more intense exposure of root acids and results in the conversion into the mobile form. In conclusion the data show an enrichment of Cd, Cs, Mn, Ni, Sr and U in the fractions I and II, except Pb which showed lesser amounts in the bioavailable fractions after 18 months. The bioavailability of Cd, Cs, Mn, Ni, Sr and U is increased with ECM treatments.

The essential elements Ca, K, Mg, Mn, S and Zn were investigated after the same method (Fig. 17). Under the influence of ECM K and Ca indicated clearly higher availabilities especially in root influenced soil zones (3 cm). Compared to the availability without plant influence in the control pot or non inoculated plant pots Mg, Mn and S showed the tendency to be lesser available in this soil region. In deeper soil zones (50 cm) without direct root contact or influence, all essential elements appeared to be lesser available (Fig. 17). The heap material with low acidic pH of 3.2-3.5 and loess loam material with pH 6–7.5 indicated very different values of element amounts. While the availability directly depends on the pH even higher amounts of elemental Sr, Ni and U in the loess loam substrate showed lower availability and therewith toxicity for organisms.

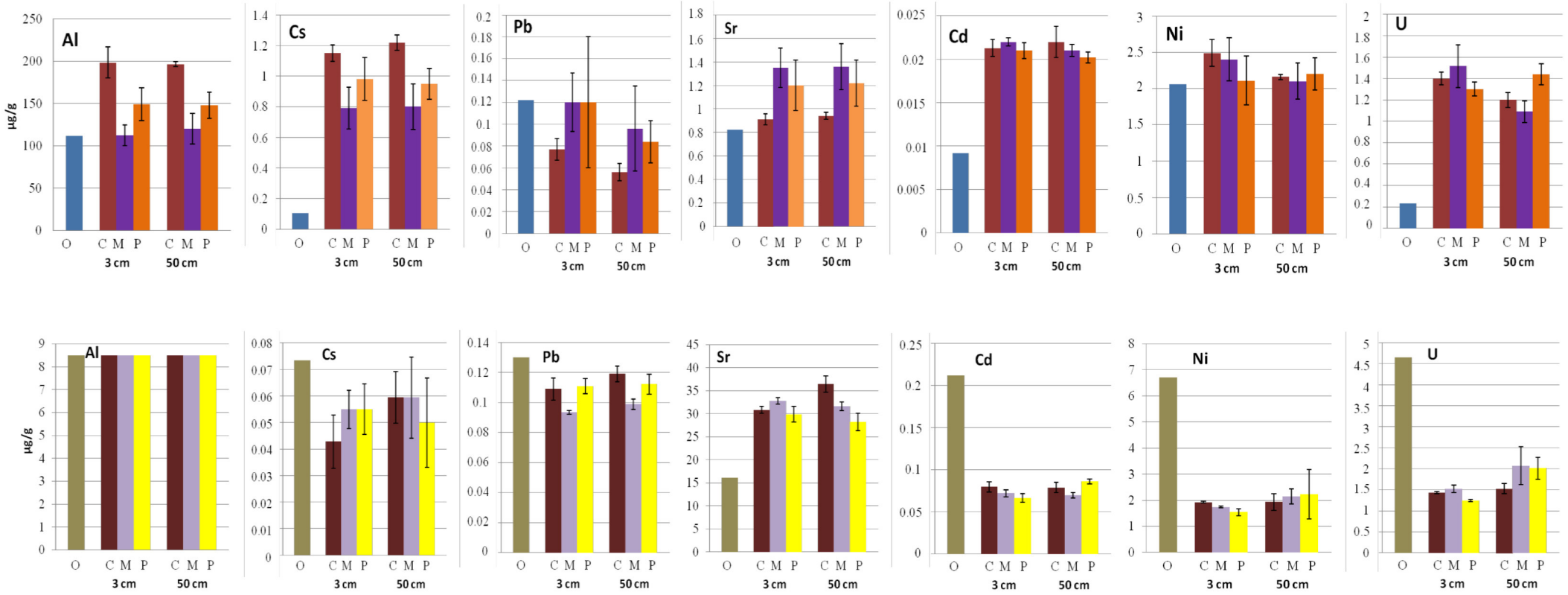

Fig. 16: **Content and alteration of non-essential elements Al, Cs, Pb, Sr, Cd, Ni and U by comparison of the initial situation (original bar in blue and green), root space area (3 cm depth) and deep soil zone (50 cm)**. Upper figures represent the heap material and the lower represent the application material loess loam. O: original initial heap material at starting point of the experiment, C: control without plants, M: ECM pot (tree + fungal partner) and P: plants (trees) without ECM fungal inoculation.

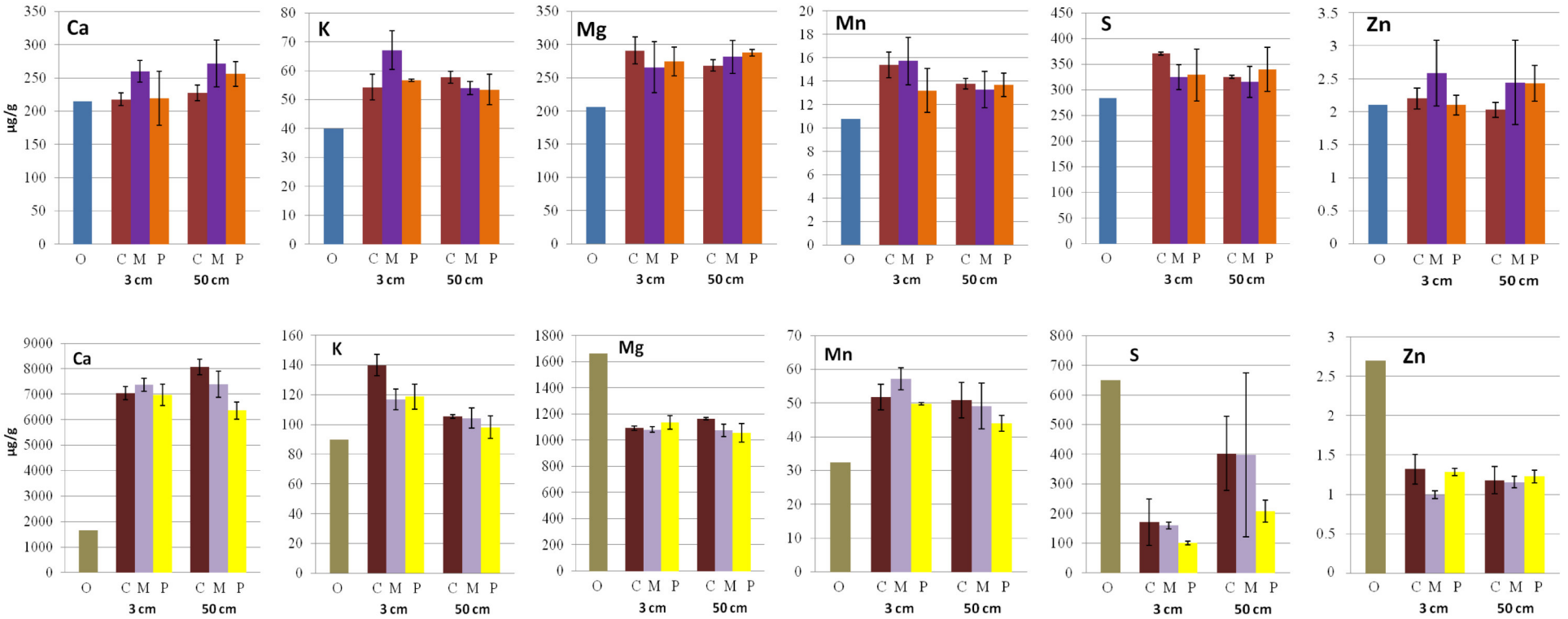

Fig. 17: **Content and alteration of essential elements Ca, K, Mg, Mn, S and Zn by comparison of the initial situation (original bar in blue), root space area (3 cm depth) and deeper indirectly, root influenced, soil zones (50 cm)**. Upper figures represent the heap material and the lower represent the application material loess loam. O: original initial heap material at starting point of the experiment, C: control without plants, M: ECM pot (tree + fungal partner) and P: plants (trees) without ECM fungal inoculation.

3.3.3. Loess material

For comparison the sandy loess loam was used for investigations. Fig. 18 shows the percentage distribution of non-essential and essential elements. Cd indicated reduced amounts in presence of ECM compared to the unplanted control in both soil depths. Cs availability in upper and downer soil regions was underrepresented. Ni showed similar distribution like Cs in the upper root influenced soil zone. With over 90% in both depths (3 cm and 50 cm) Sr showed the major proportion with and without ECM and root contact. In deeper soil zones with indirect influence of ECM and plant roots the amount of Sr was a little less. With 5% U showed a tendency to have larger amounts in the deeper indirectly ECM and root influenced soil zones.

The essential elements of the sandy loess loam, showed different behavior compared to the heap material. The element with the main proportion represents Ca with over 80% in both soil depths. A little less Ca amounts could be measured in deeper soil zones. Mg amounts of around 10% were found, with a tendency to decrease in deeper soil zones, especially under influence of ECM. Larger amounts of S appeared in the deeper soil part, especially under influence of ECM while K showed the lowest proportions in all soil zones (Fig. 18). All in all the non-essential elements in the sandy loess loam indicated inhomogeneous and individual element distribution after the experimental set-up of 1.5 years.

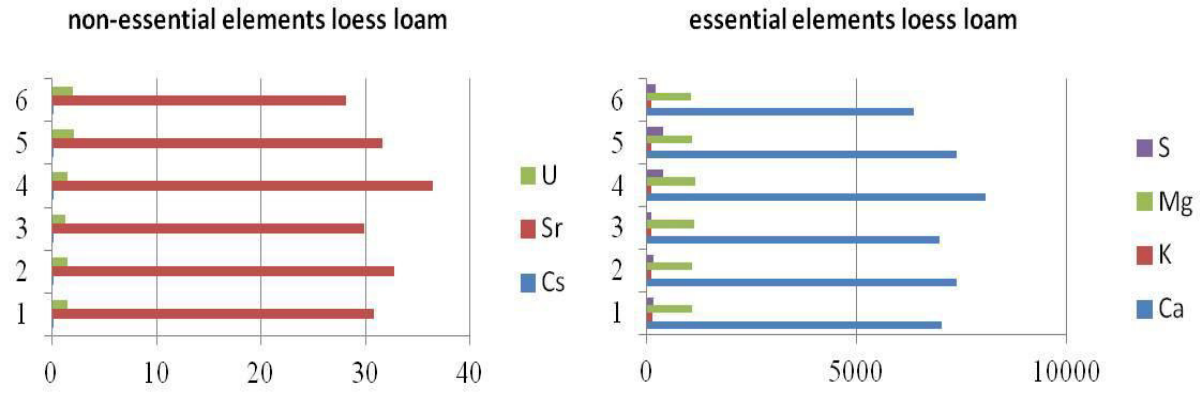

Fig. 18: **Content of elements in loess loam after experimental treatment**. 1-3: root space 3 cm soil depth with 1: control without plants, 2: ECM plants, 3: plants without ECM. 4-6: deep zone 50 cm soil depth without roots with 4: control without plants, 5: ECM plants, 6: plants without ECM.

By the help of a logarithmic scaling of the non-essential elements' proportions of the sandy loess loam material (Fig. 19) the influence of ECM varied compared to bioavailable elements of the heap material (Fig. 15). In general plants with as well as without ECM influenced the availability of elements in a neutral pH substrate compared to the control

sample (Fig. 19). The main proportion of non-essential elements was represented by Cs and Sr. In comparison to the control pots it is conspicuous that the elements U, Cd, Ni were underrepresented or lesser available especially in substrates with plants (Fig. 19). Pb was in general very little available in the sandy loes loam.

The essential elements showed clear differences. The Ca availability increased over the experimental period more than 3 times in both soil depths under influence of ECM (Fig. 19). Furthermore Ca indicated a tendency to accumulate in deeper soil zones when no plant cover was present. While S showed the tendency to accumulate in deeper soil zones, too, the influence of ECM to S availability in the deeper soil regions (Fig. 19) seems disputable and not clearly depending on the ECM. The elements K, Mg and Mn indicated no clear deviation in availability during the period of the experiment.

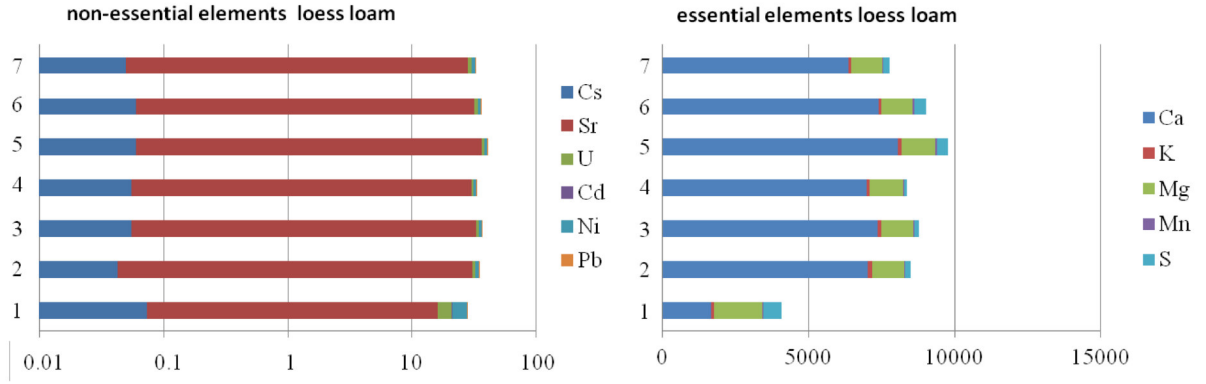

Fig. 19: **Logarithmic scaling of the y-axis of proportions of non-essential and essential elements of loess loam after experimental treatment in total**. 1: original untreated soil. 2-4: root space 3 cm soil depth with 2: control without plants, 3: ECM plants, 4: plants without ECM. 5-7: deep zone 50 cm soil depth without roots with 5: control without plants, 6: ECM plants, 7: plants without ECM.

Once more the essential elements of the loess loam showed an individual and inhomogeneous pattern of distribution (Fig. 19). While the availability of Ca, K and Mn was clearly increased in both soil depths, Mg and S indicate contrary results compared to the initial material. Ca showed a bioavailable increase in both depths with a little tendency to decrease in the deeper soil zone under influence of ECM. The distribution pattern of Ca resembles Sr (Fig. 16 and 17) with higher availability under presence of ECM and lesser availability without ECM. Furthermore K showed, next to the higher availability in general much lesser amounts in the upper ECM root influenced soil zone and lesser but still reduced availability in the deeper soil zone. The highest amount of K was found in the control pots without plants. Similarly Mn showed a generally higher availability compared to initial material but higher bioavailability especially in the upper root influenced soil zone with ECM.

Mg indicated a minimally less availability with ECM in upper and deeper soil zones whereas S clearly occured much lesser in the upper soil zones (Fig. 16).

In conclusion the elemental data of the fractions I and II of loess loam analyses showed a depletion of Cd, Cs, Ni, Pb and U after 1.5 years, except for Sr and Mn which increased in bioavailable fractions. The statistic normal distribution analysis of the mesocosm experiment data induced different results. By comparison of bioavailable non-essential and essential elements in soil with and without ECM with two different substrates and depths, a variety of significances occurred. The non-essential element Al indicated a significant elemental change in both soil depths. The essential elements K and Mg showed a significant elemental change in the upper soil depth within the root space in case of heap material (Tab. 7). The loess loam, on the other hand, showed a significant change of the non-essential elements U, Pb and the essential element S in the upper soil zones (Tab. 7) with root contact.

Tab. 7: Comparison of bioavailable elements in soil with and without ECM from mesocosms with two different soil types and depths. Root space (3 cm), deep zone with indirect or inconsistent root influence (50 cm). Signature: + significant change of element concentration, - no significant change in element concentration.

	Loess 3 cm	**Loess 50 cm**	**Heap substrate 3 cm**	**Heap substrate 50 cm**
Ca	-	-	-	-
K	-	-	+	-
Mg	-	-	+	-
S	+	-	-	-
Cs	-	-	-	-
Sr	-	-	-	-
U	+	-	-	-
Al	-	-	+	+
Pb	+	-	-	-

The statistical comparison of element data of the heap substrate between control and ECM treatment (Tab. 8) showed a significant change of S (around 14%) in the root soil zone and Cs in both soil depths. The elemental Cs content was in both depths reduced by one third compared to control sample. The loess loam showed a more differentiated outcome. In the root influenced soil zone K was 20% reduced in comparison to the control samples. Pb indicated significant differences, too. In the deeper soil zone without or only indirect root influence U was over 30% more available.

Tab. 8: Comparison of bioavailable elements from mesocosms within control soil and with ECM. Signature: + significant change of element concentration, - no significant change in element concentration.

	Loess 3 cm	Loess 50 cm	Heap substrate 3 cm	Heap substrate 50 cm
Ca	-	-	-	-
K	+	-	-	-
Mg	-	-	-	-
S	-	-	+	-
Cs	-	-	+	+
Sr	-	-	-	-
U	-	+	-	-
Al	-	-	-	-
Pb	+	-	-	-

In Tab. 9 the significant elemental changes between the two soil depths of the heap substrate can be seen. K, Mg, Mn and U showed differences between upper and deeper soil depth in presence of plants colonized with ECM. While S and U already showed differences in the control samples.

Tab. 9: Comparison of bioavailable elements from mesocosms within the different soil depths: root space (3cm) and deeper soil zone (50 cm) of heap substrate. Signature: + significant change of element concentration, - no significant change in element concentration.

	Control 3 cm / 50 cm	With ECM 3 cm / 50 cm	Without ECM 3 cm / 50 cm
Ca	-	-	-
K	-	+	-
Mg	-	+	-
S	+	-	-
Cs	-	-	-
Sr	-	-	-
U	+	+	-
Mn	-	+	-

By comparing the loess loam treatments control, with and without ECM in two different depths, the essential elements Ca, K, Mg and non-essential element Sr showed significant changes between upper and deeper soil zones only without any plant root, microorganism or other organism influence (Tab. 10). And moreover the non-essential elements Cd and Ni indicated significance without ECM between the upper and deeper soil regions (Tab. 10).

Tab. 10: Comparison of bioavailable elements from mesocosms of loess loam within different soil depths: root space (3 cm) and deeper soil zone (50 cm). Signature: + significant change of element concentration, - no significant change in element concentration.

	Control 3 cm / 50 cm	With ECM 3 cm / 50 cm	Without ECM 3 cm / 50 cm
Ca	+	-	-
K	+	-	-
Mg	+	-	-
S	-	-	-
Cs	-	-	-
Sr	+	-	-
U	-	-	-
Cd	-	-	+
Ni	-	-	+

3.3.4. Effect of metals on plant growth

The different substrate types as well as the different depths show a variety of the amount of bioavailable elements. To quantify root and therewith plant vitality, the root length of young trees of *Picea abies* and *Pinus sylvestris* of the mesocosm experiments with heap substrate was measured (Fig. 20).

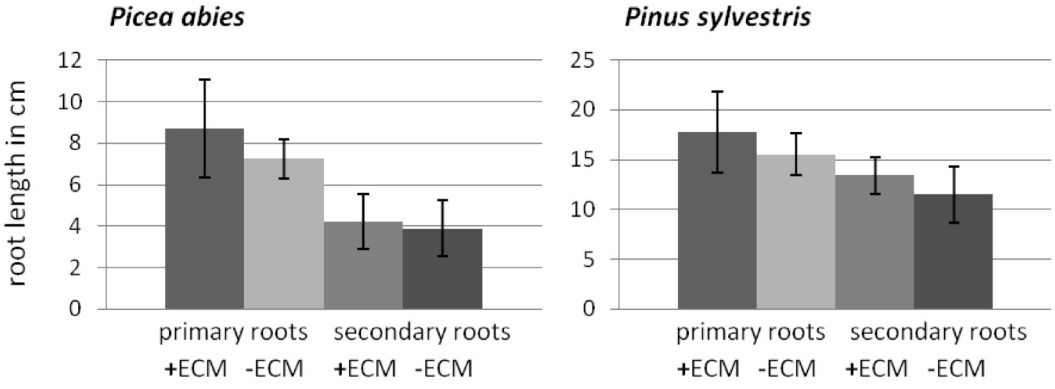

Fig. 20: **Average of primary and secondary root length of *Picea abies* and *Pinus sylvestris* from mesocosms with heap soil substrate after 1.5 years**.

Primary as well as secondary roots without ECM indicated lesser lengths compared to roots with ECM (Fig. 20). *P. abies* mainly showed lesser lengths and reduced primary branches (2-5) per root system compared to mycorrhized roots with 3-7 branches per each root system (Fig. 20). In the case of *P. sylvestris* the length of primary and secondary roots differed around ¼. Mycorrhized root systems generated 5-8 branches with many little additional branches compared to solely 6-7 branches and missing development of very fine

branches without ECM (Fig. 20). All in all after 1.5 years of mesocosm experiments *P. sylvestris* held the main proportion of the tree vegetation.

3.4. Protection of fungal hyphae through glutathione S-transferase activity

The activity of the glutathione S-transferase (GST) enzyme was investigated to detect how the ECM symbiosis handles toxic substances intracellularly. The GST key role was examined in three different axenic systems:

- in pure fungal cultures of early ECM colonizers *P. tinctorius* and *P. involutus*, and of the late ECM colonizer *T. vaccinum* (Fig. 21);
- in young tree seedlings of *P. abies* and *P. sylvestris* shortly after germination in the beginning ECM symbiosis;
- in ECM symbiosis in sandwich cultures.

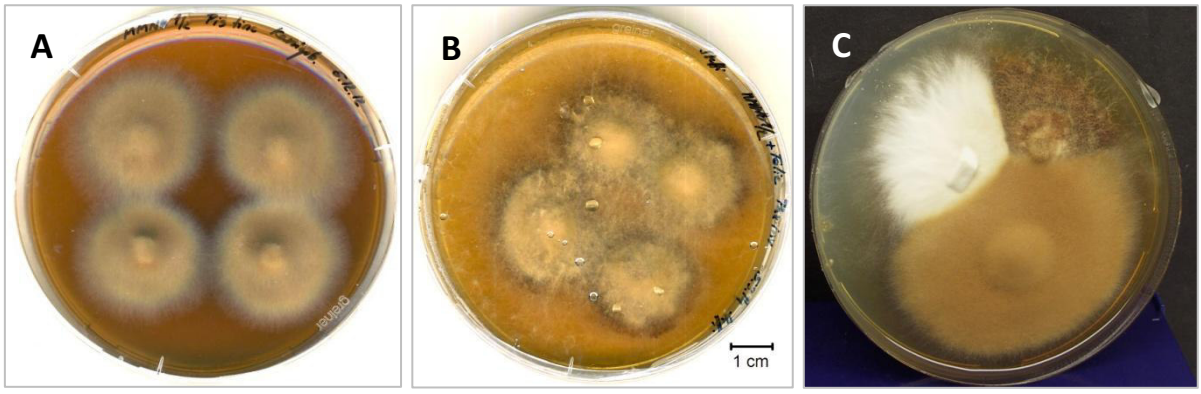

Fig. 21: **Axenic cultures of ECM fungi on MMNb ½ medium**. Pure cultures of **A** *P. tinctorius* and **B** *P. involutus* after 2 months. **C** Three ECM fungi (clockwise): *P. involutus* (brown), *P. tinctorius* (light brown) and *T. vaccinum* (white) grown together with 0.5 mM Cs_2SO_4 treatment after 3 months.

Mycelium of *P. tinctorius* showed the highest GST activity with 11 µmol/g/min (Fig. 22) in treatments of 1 mM $NiCl_2$, 7 µmol/g/min in 40 mM $SrCl_2$ and 6 µmol/g/min in 1 mM $PbCl_2$. $NiCl_2$ seems to be the most stressing metal salt for *P. tinctorius*. Even low concentrations of this metal salt indicated high reactions (Fig. 22). Very little GST activity (less than 0.5 µmol/g/min) was measured in 10 mM CsCl (Fig. 22). This indicates, again, very high tolerable values for this element besides the already mentioned Sr. Also the mycelium of fruiting bodies of *P. tinctorius* has the potential to accumulate Cs in great amounts (see BCF values of 12; Tab.1). By statistic analysis of GST activity data of *P. tinctorius* (Fig. 22) no

significant difference between the HM and radionuclide treatments was observed ($\chi^2 = 3.757$; d.f. = 3; p = 0.05). While 1 mM $PbCl_2$ samples had the highest (9.00), the 10 mM CsCl samples had the lowest (1.00) mean rank.

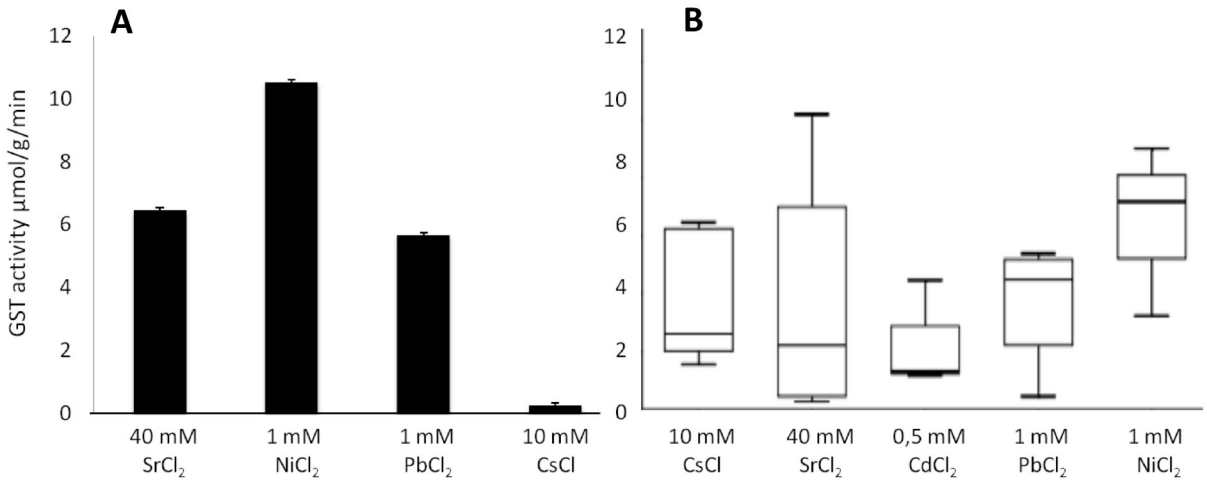

Fig. 22: **GST activity of HM/Ra treated mycelium of *Pisolithus tinctorius***. **A** Bar diagram. **B** Statistical analysis.

Cytosolic GST activity of *P. involutus* was investigated, too (Fig. 23). Again, the most intense signal was registered from treatments with 1 mM $NiCl_2$: 18 µmol/g/min. This corresponds with the low fruiting body BCF values of 3 and indicates high stress for the fungal cell. But also intense signals of 9 µmol/g/min were detected in 40 mM $SrCl_2$ treatments. The GST activity of *P. involutus* grown in combination with *P. tinctorius* on 40 mM $SrCl_2$ media gave a much lesser signal of 3 µmol/g/min, while the control value of pure MMNb ½ is only 2 µmol/g/min. The GST activity of the 1 mM $PbCl_2$ treatment was 7 µmol/g/min and of the 15 mM CsCl 3 µmol/g/min. These Cs measurements correspond with very high BCF values of 66 for Cs of the fruiting body mycelium of *P. involutus* (Tab. 1). It indicates, again, high tolerable levels for this radionuclide, even in relation to cell detoxification abilities of this ECM. The statistic validation of *P. involutus* samples with and without HM and radionuclides resulted without significant difference ($\chi^2 = 1.405$; d.f. = 4; p < 0.05).

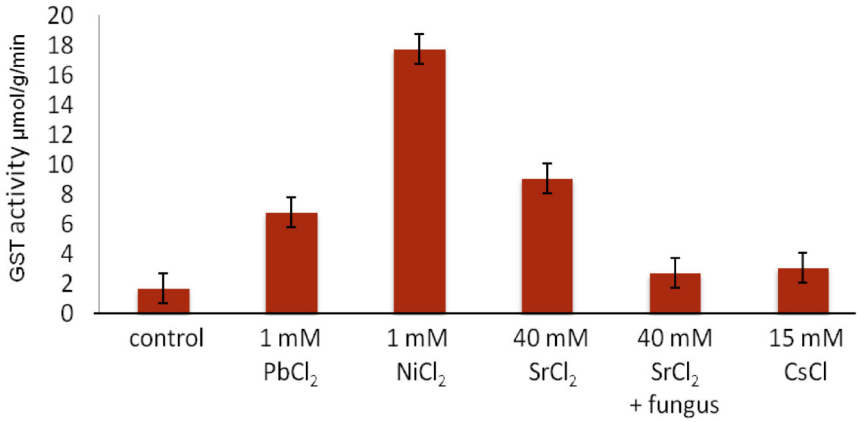

Fig. 23: **GST activity of mycelium of *Paxillus involutus*, with *P. tinctorius* co-culture in $SrCl_2$ treatment**.

GST activity of fungal mycelium of late colonizer and specialist *T. vaccinum* was measured (Fig. 24). In general the GST activity of *T. vaccinum* was relatively low along the used metal concentrations with inconspicuous or normal growth rates in all treatments. Even higher values did not reach 3 µmol/g/min GST activity compared to clearly higher amounts of the already described early colonizer and generalist ECM fungi *P. tinctorius* and *P. involutus*. Higher GST activity values were reached by *T. vaccinum* mycelium cultures in combination with *P. tinctorius*: approximately 2.7 µmol/g/min in 1 mM $PbCl_2$ and 40 mM $SrCl_2$. Seperate fungal cultures of *T. vaccinum* indicated GST activity values about 2 µmol/g/min in 1 mM $PbCl_2$. The GST activity in 1 mM $NiCl_2$ showed values of 1.6 µmol/g/min. The lowest values (0.6 µmol/g/min GST activity) were found in 0.4 mM $CdCl_2$ and 0.4 mM $CdSO_4$ concentrations. These data assume a much higher tolerance of *T. vaccinum* mycelium in all examined metal concentrations and imply high cellular tolerance thresholds for metal substances.

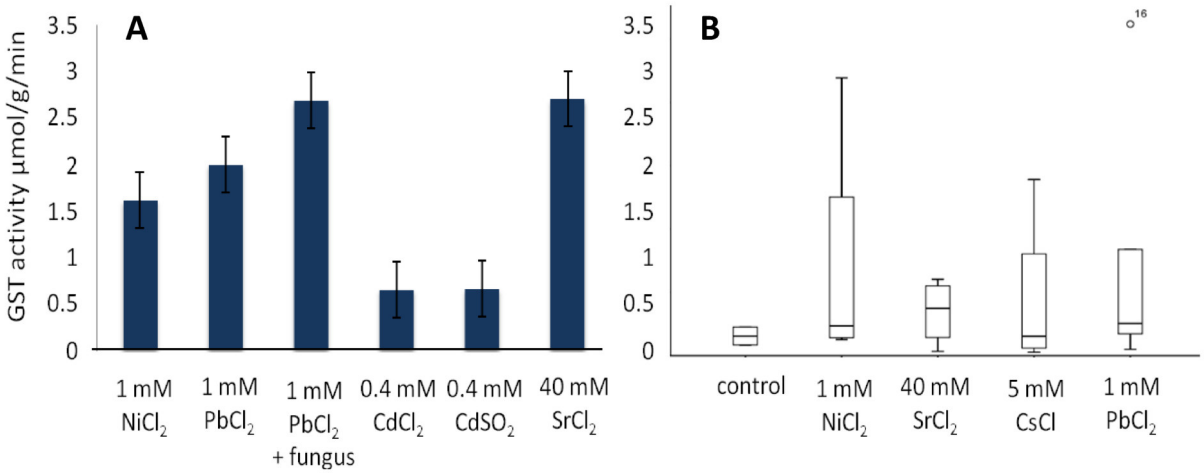

Fig. 24: **GST activity of HM/Ra treated mycelium of *Tricholoma vaccinum***. **A** Bar diagram with *P. tinctorius* co-culture in $PbCl_2$ treatment. **B** Box blot presentation of the data. (° outlier over 1.5 of the interquartile range).

For statistic analysis of *T. vaccinum* data the non-parametric Kruskal-Wallis test was used (Fig. 24). None of the interquartile ranges of the HM and radionuclide treatments differed. The control sample showed the lowest (7.50) and the 1 mM $PbCl_2$ the highest (11.83) mean rank in comparison. The statistic analysis of *T. vaccinum* resulted in no significant difference between the HM and radionuclide treatments ($\chi^2 = 4.346$; d.f. = 4; $p < 0.05$).

By comparing all three ECM fungal data (Fig. 25) *P. involutus* showed the highest values, *P. tinctorius* moderate and *T. vaccinum* low GST activities. This assumption is confirmed by statistic analysis of the non-parametric Kruskal-Wallis test. A significant difference occurred in the GST activities in between the species ($\chi^2 = 12.700$; d.f = 2; $p > 0.002$). While *P. tinctorius* and *P. involotus* did not significantly differ in their median GST activity ($U = 141.0$; $n_1 = 14$; $n_2 = 23$; $p > 0.05$), *T. vaccinum* and *P. tinctorius* ($U = 73.0$; $n_1 = 21$; $n_2 = 14$; $p < 0.05$) and *T. vaccinum* and *P. involotus* ($U = 92.0$; $n_1 = 21$; $n_2 = 23$; $p < 0.05$) differed significantly. Therefore the GST activity of late colonizer ECM fungus *T. vaccinum* differed significantly in comparison to the two other early colonizer ECM species (Fig. 25).

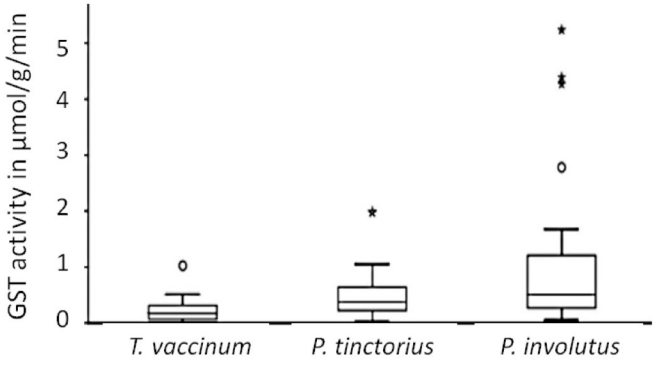

Fig. 25: **Comparison of GST activities of mycelium of *T. vaccinum* with *P. tinctorius* and *P. involutus***. Significant difference occurred between the GST activities of the two early colonizer fungi *P. tinctorius* and *P. involutus* compared to the late colonizer fungus *T. vaccinum*, but no between the two early colonizer fungi. (° outliers over 1.5 of interquartile range, * data points more than 3 interquartile ranges from the highest interquartile range value).

Furthermore young tree seedlings of *P. abies* and *P. sylvestris* were investigated during and direct after the germination with and without ECM fungal addition (Fig. 26). They grew exclusively on germination agar. This represents the very early signalling of GST activity in ECM formation grown on HM substrates.

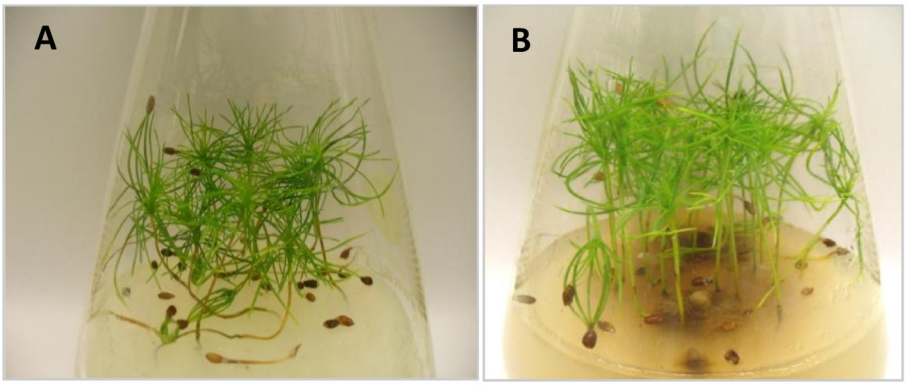

Fig. 26: **Young tree seedlings of *P. sylvestris* on germination agar**. **A** after 3 months, **B** ECM with *P. involutus* after 3 ½ months.

After separation of the trees in crown and stem (C+S) and root and mycelium (R+M) a two grouped measurement of the GST activity was taken (Fig. 27). The values were not homogenous with individual results. The control data of *P. sylvestris* yielded in more than 2 times higher GST activities compared to the control data of *P. abies* (Fig. 27). By adding the three fungi *P. tinctorius*, *P. involutus* and *T. vaccinum* to *P. sylvestris* the GST activity in the control sample was clearly decreased. Generally the GST activity in *P. sylvestris* was higher

in C+S compared to the root. This was strengthened by the addition of fungi and their influence. The GST activity of *P. abies* was already in the respective control media much lower compared to *P. sylvestris*. Whereby the GST activity in C+S was three times lower compared to the GST activity in the root. Without influence of HM the GST activity in the control plants of *P. abies* and *P. sylvestris* seems to be opposing. Higher GST activities in C+S of *P. sylvestris* and the roots of *P. abies* as well as lower GST activities in the roots of *P. sylvestris* and C+S of *P. abies* underlined this outcome.

By adding 0.1 mM $CdCl_2$ the GST activity increased clearly in C+S from 2 µmol/g/min in the control medium up to 11 µmol/g/min in HM treatments. The GST activity of the roots (R) was markedly lower: 3 µmol/g/min in 0.1 mM $CdCl_2$ media of *P. abies* (Fig. 27). Whereby the GST activity of *P. abies* in 1 mM $PbCl_2$ media tends to be higher in C+S samples (8 µmol/g/min) compared to the R (7 µmol/g/min). It showed a 4 times higher GST activity in C+S, but only a minimal higher value in the R compared to the control sample. These GST activity data of *P. abies* indicated a higher stress potential in C+S under HM influence, while the stress potential level tends to stay moderately in R. Subsequently it is to mention that the GST activity of *P. abies* was largely increased under influence of HM in C+S compared to the roots. The effect of HM treatments on *P. sylvestris* was different. So under influence of HM the GST activity increased mainly in the R and decreased in C+S. This fact becomes more clear under influence of fungi (Fig. 27).

1 mM $PbCl_2$ influenced *P. sylvestris* C+S a bit less (5 µmol/g/min) compared to the R (6 µmol/g/min), while the control media showed values of 8 µmol/g/min for C+S and 7 µmol/g/min for the roots. Under influence of 1 mM $CdCl_2$ with *P. involutus* this effect seen in 1 mM $PbCl_2$ was strengthened. Accordingly the GST activity of C+S was 5 µmol/g/min and 7 µmol/g/min in R+M. Compared to the control *P. sylvestris* with the three ECM fungi *P. tinctorius*, *P. involutus* and *T. vaccinum* the GST activity showed opposite values (Fig. 27). So in the control plants *P. sylvestris* C+S with fungi, the GST activity was higher (6 µmol/g/min) compared to C+S with fungus and HM (5 µmol/g/min). The GST activity of control R+M with fungi was lower (3 µmol/g/min) compared to R+M with fungus and HM treatments (6 µmol/g/min). These data indicated higher cytosolic GST activity signals under HM treatments in the roots compared to the control that showed lower GST activities in the roots but higher GST activities in C+S. *P. abies* and *P. sylvestris* showed opposite GST activities when grown with HM. Both trees indicated individual GST activities – in *P. abies* higher values in C+S but lower in R; in *P. sylvestris* by trend lower GST values in C+S and R.

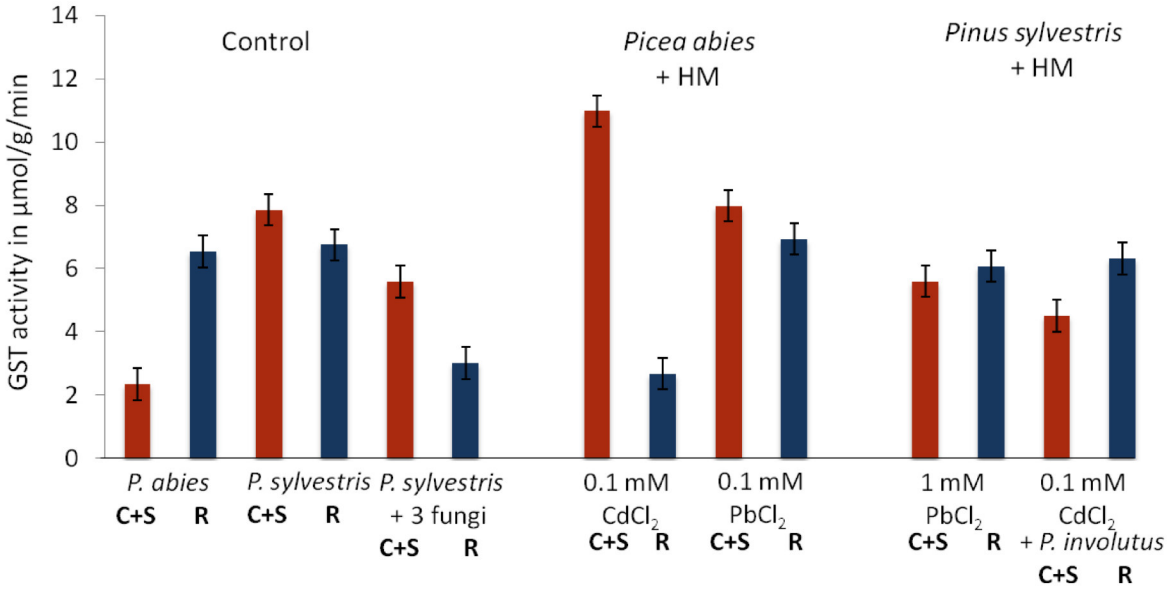

Fig. 27: **GST activity of devided fresh young trees from germination medium in addition with or without different HM and in symbiosis with ECM fungi *P. tinctorius*, *P. involutus* and *T. vaccinum***. Red bars indicate crown and stem (C+S) and blue bars indicate roots (R) with mycelium of the trees.

However for the two groups C+S (crown and stem) and R+M (roots and mycelium) the non-parametric Kruskal-Wallis test of the parted young trees in germination agar (Fig. 28) revealed no significant GST activity of the samples grown with or without HM ($\chi^2 = 0.245$; d.f. = 1; $p < 0.05$). But still the shown data indicate higher values in crown and stem.

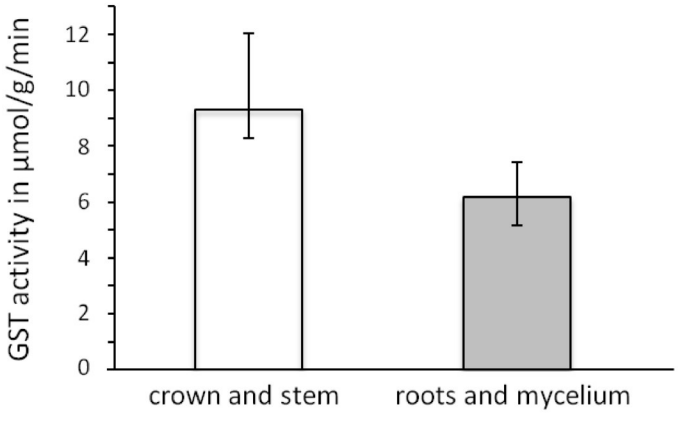

Fig. 28: **Kruskal-Wallis test of GST activities of young ECM pine and spruce trees from germination medium in all treatments and with *P. tinctorius*, *P. involutus* and *T. vaccinum***. The difference of GST activities between the parts of the trees showed no significant difference between the two groups. The column values represent the means for the groups with ± SE (standard error) bars.

Furthermore the GST activity of fresh sandwich culture (Horan *et al*. 1988) samples was measured (Fig. 29). The advantage of axenic ECM co-cultures is the explicit interaction between the two partners and thus its positive mutual support. For testing the GST activity the plant-fungus system was again divided into the former two groups: C+S (crown and stem) and R+M (roots and mycelium).

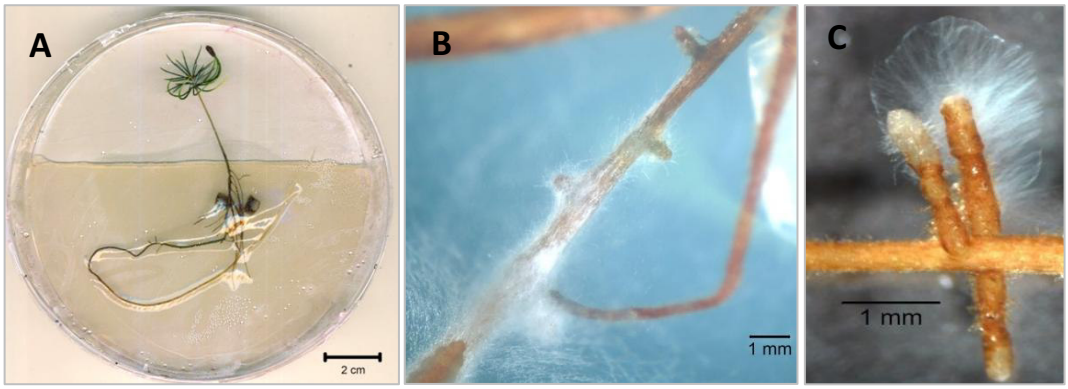

Fig. 29: **Sandwich culture of ECM partners on MMNa medium. A** *P. abies*, *P. involutus* and *T. vaccinum* after 3 months. **B** *P. sylvestris* and *T. vaccinum* with ECM short roots with 1 mM $PbCl_2$ after 5 months. **C** *P. abies* and *T. vaccinum* with 40 mM $SrCl_2$ after 7 months.

The sandwich-culture systems with *P. abies* combined with either *P. involutus*, *P. tinctorius* or *T. vaccinum* were again tested with different HM and radionuclide salts: 1 mM $PbCl_2$, 40 mM $SrCl_2$, 10 mM CsCl and 0.4 mM $CdCl_2$. Different GST activity results are shown in Fig. 30. The control culture with *P. involutus* showed very high GST activity in C+S (9 µmol/g/min) compared to the R+M (0.4 µmol/g/min). *P. abies* in 1 mM $PbCl_2$ and in symbiosis with *T. vaccinum* showed GST activity values of 4 µmol/g/min in C+S and 2 µmol/g/min in R+M. The GST activity in 40 mM $SrCl_2$ was relatively low: 0.5 µmol/g/min in C+S and 1 µmol/g/min in R+M. 10 mM CsCl *P. abies* with *P. tinctorius* showed higher GST activity levels in C+S: 4.5 µmol/g/min. In comparison the R+M had only a value of 1 µmol/g/min. On the contrary *P. abies* in combination with *T. vaccinum* showed opposite results: 1 µmol/g/min in C+S and 3 µmol/g/min in R+M. Also higher GST activity values were measured with 0.4 mM $CdCl_2$ (Fig. 30). *P. abies* in combination with *P. tinctorius* showed 6.5 µmol/g/min in C+S and 5.5 µmol/g/min in R+M. GST activity data from sandwich cultures showed higher or lower results depending on HM and radionuclide but also on the fungal partner. By trend the cytosolic GST activity was higher in C+S.

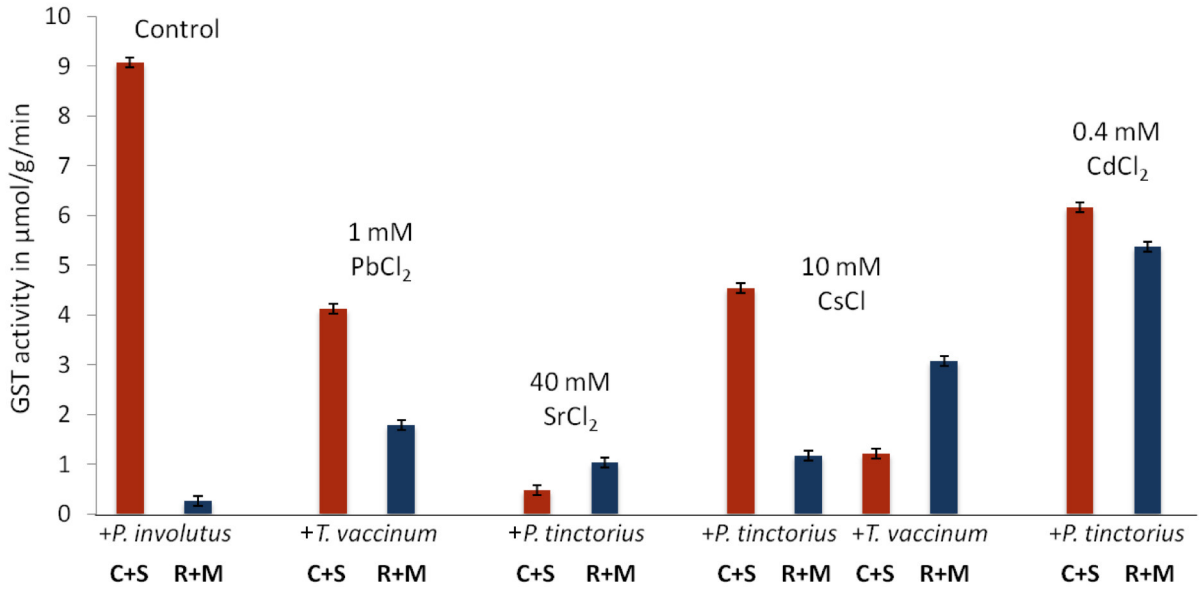

Fig. 30: **GST activity of ECM *Pices abies* - *P. tinctorius*, *P. involutus*, *T. vacciunm* sandwich-cultures**. Red indicates crown and stem (C+S), blue indicates root and mycelium (R+M). The GST activity in C+S by trend was higher compared to the R+M, except for results of 40 mM $SrCl_2$ (opposite reactions).

C+S was compared to data of R+M by means of the paired-sample t-test. It indicated a significance in the C+S GST activity ($t = 1.122$; d.f. = 11; $p > 0.5$) of young *P. abies* seedlings with or without ECM fungus (Fig. 31). An overlap of the standard error of the two samples can be seen in the means (difference of 1.77 µmol/g/min). For testing significance with or without HM and radionuclide addition the Kruskal-Wallis test was chosen. No significant difference was found in the GST activity of the samples with or without HM ($\chi^2 = 1.692$; d.f. 1; $p < 0.05$) (Fig. 31). Again an overlap in the standard errors appeared between the HM and non HM treated samples. Most of the samples showed differences of value, but most statistic analysis did not result in significance.

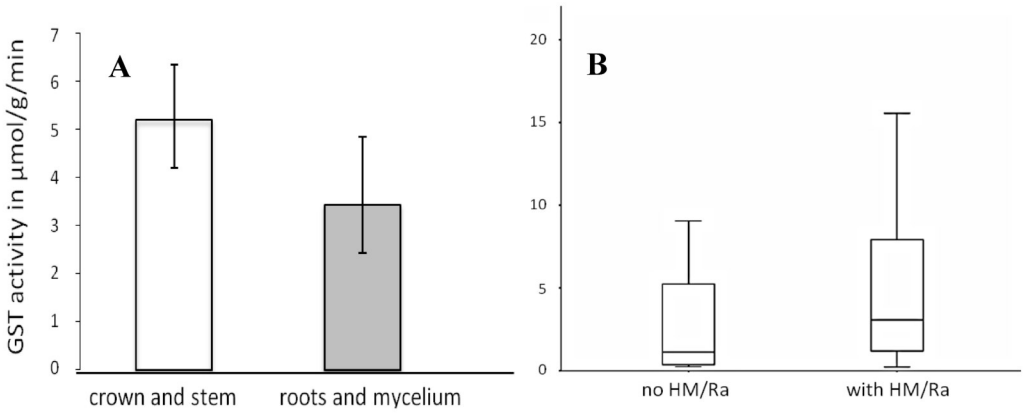

Fig. 31: **GST activity of tree parts. A** from sandwich cultures. The paired-sample t-test resulted into a significant difference of GST activity between the groups C+S (crown and stem) and R+M (roots and mycelium). **B** Comparison of GST activity of sandwich culture samples grown on media with and without HM/Ra. No significance was found in between the two groups.

3.5. Protection of fungal tissue through production of guttation droplets

In fungal cultures, especially under heavy metal treatment, the production of guttation droplets (GD) was observed. To investigate a possible function of protecting mycelia, different fungi were investigated more in detail.

GD consist of more or less pigmented, watery and water soluble droplets. The observations showed GD probably as result of a general stress reaction seen by an extensive drop formation. However depending on the fungal species a variety of coloration reaching from colorless, watery or oatmeal to brown were observed.

Approximately 35% of the fungal cultures of *P. tinctorius*, *P. involutus*, *T. terreum*, *T. vaccinum*, *S. commune* FSU: 3214 x FSU: 2896 and *S. commune* Δ*Ku8070* T 14(4) x C6 showed guttation on aerial mycelium after 1 to 3 months of incubation with amounts differing according to species and strains (Fig. 32). The saprophyte *S. commune* wt and mutant strain *S. commune* Δ*Ku8070* T 14(4) x C6 indicated a much larger amount of GD volume in comparison to ECM fungi with early colonizers *P. tinctorius* and *P. involutus* as well as low amounts or even absence of GD on late colonizer *T. vaccinum* or *T. terreum* cultures (Tab. 11).

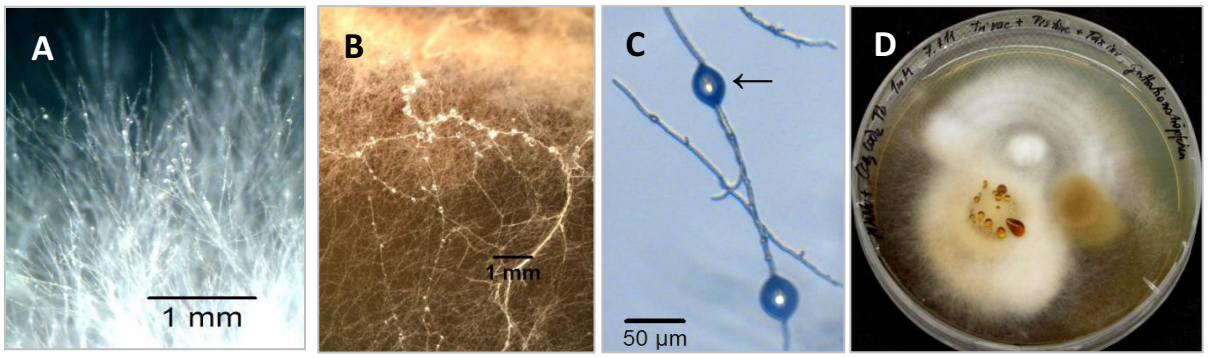

Fig. 32: **Guttation on hyphae of basidiomycetes. A** on hyphal tips of *P. tinctorius*, after 2 weeks. **B** along hyphal strands of *P. involutus*, after 1.5 months. **C** on septa of *S. commune*, after 1 week. **D** on aerial mycelium of *P. tinctorius* together with *P. involutus* and *T. vaccinum* in co-culture, after 5 months.

All basidiomycetes excreted most GD under the influence of day light and 1 mM $PbCl_2$ (Tab. 11). The early colonizer fungi *P. tinctorius* and *P. involutus* are well known from literature for their high tolerance within toxic substances like metals (Deacon 2006). However individual effects of HM/Ra concentrations were seen. Lower tolerance against $CdCl_2$ was shown for the early colonizer ECM fungi, indicated by small colony diameters, fungal mycelium growth and low amounts of GD (18 µl, after a longer time; see Tab. 11). To enable fungal growth the $CdCl_2$-concentration had to be lowered during the experiment from 0.2 mM to 0.1 mM. Both early colonizers, *P. tinctorius* and *P. involutus*, showed tendencies to tolerate 1 mM $PbCl_2$ and 40 mM $SrCl_2$ indicated by large growth rates. In the case of *P. tinctorius* 24 h artificial light as well as addition of 0.1 mM $CdCl_2$ induced decreased fungal culture growth. The occurrence of primordia was associated with higher amounts of GD on *S. commune*, too.

Most GD occurred under influence of 0.5 mM and 1 mM $PbCl_2$: around 300-500 µl/l. Pigmented GD of both early colonizers' mycelium cultures, *P. tinctorius* and *P. involutus*, were collected, while *P. involutus* produced the most GD. Partially pigments could be separated by centrifugation. Most of the GD occurred after 4-6 months on ageing cultures of ECM fungi. The main amounts of GD on *S. commune* were found in ageing cultures and at lamellae of fruiting bodies. Particularly high amounts appeared on their primordia, the pre-stages of the fruiting bodies.

Tab. 11: Characterisation of guttation droplets (GD) after 40 d, quantity of GD: * 1-5; ** 5-15; *** > 15 (modified and with additions after Sehrt 2013).

Species	Cultivation	First occurrence of GD`s	Ø of droplets	Total volume of GD from fungal cultures	Color	Quantity	Remark
Pisolithus tinctorius	Day light, MMNb ½	10 d	1.5 mm	280 µl	transparent / brown	**	
Paxillus involutus	Day light, MMNb ½	12 d	2 mm	418 µl	transparent / dark brown-black	**	GD mainly transparent, 2 replicates up to 2 mm size and dark brown-black pigmented.
	+ 1 mM $PbCl_2$		0.07 mm	18 µl	transparent	*	
	+ 0.1 mM $CdCl_2$						
Tricholoma vaccinum	Day light, MMNb ½	9 d	0.05 mm	7 µl	transparent	**	
	+ 1 mM $PbCl_2$					*	After 18 d no GD occurred anymore.
	+ 0.2 mM $CdCl_2$						
Tricholoma terreum	+ 40 mM $SrCl_2$	24 d	0.06 mm	11 µl	transparent	*	
Schizophyllum commune dikaryon, wt	Day light, MMNb ½,	5 d	1.6 mm	214 µl	transparent	***	In ageing mycelium largest numbers of GD, after 20 d large GD at primordial.
	24 h artificial light, MMNb ½		0.11 mm	69 µl		*	GD occurred mainly at younger mycelium and primordia
	+ 1 mM $PbCl_2$		1.2 mm	91 µl		**	
	+ 0.2 mM $CdCl_2$	11 d	0.2 mm	418 µl		*	
Schizophyllum commune dikaryon, mutant	Day light, MMNb ½	5 d	2.2 mm	557 µl	transparent	***	
	24 h artificial light, MMNb ½		0.15 mm	176 µl		*	
	+ 1 mM $PbCl_2$		1.9 mm	359 µl		***	
	+ 0.2 mM $CdCl_2$		0.09 mm	38 µl		**	
	+ 40 mM $SrCl_2$	11 d	0.12 mm	90 µl		**	
	+ 0.002 mM Azoxystrobin	5 d	2 mm	418 µl	transparent / yellow	***	GD`s initially overridingly at young mycelium, within ageing equally distributed over mycelium.

By means of the thin layer chromatography an analysis of the consistency of the GD of *S. commune* wt and mutant strains was done (Fig. 33). It was used to prove a cytosolic origin of the GD. The chemical analysis of GD of the *S. commune* wt and mutant strain within a thin layer chromatography was possible, because both strains produced quantifiable amounts of minimum 100 µl volume after 2 months. The advantage of this method was, that only very low volumes of 50 µl GD were needed. In all samples the amino acid, L-glutamine, and within the *S. commune* Δ*Ku8070* T 14(4) x C6 mutant strain with and without azoxystrobin the sugar D-ribose was proven.

Within the treatment with ninhydrine reagent a red coloration of the solvent route occurred in the amino acid prove. But normally the ninhydrine reaction results in a coloration into blue violet. After Stahl (1969) complex formations of Cu, Cd or Ca cations can shift the coloration into red. Since the macroelement Ca is present in the cytosol it most likely reacts as co-factor for enzymes. This could be an additional hint for the cytosolic origin of the GD. Within this framework, positive pentose ribose and amino acid glutamine content in GD occurred. *S. commune* mutants showed a considerable amount of D-ribose under influence of 0.002 mM azoxystrobin, the cell respiration affecting strobilurin. Less intensity was found in samples without any treatment (Fig. 33). The dikaryotic wt exuded very low amounts of D-ribose. The amounts of L-glutamine in the *S. commune* mutant as well as in the wt were exuded in high amounts in the GD. Highest amounts were detected in *S. commune* mutant samples treated with strobilurin but also in those samples without strobilurin influence (Fig. 33). The *S. commune* dikaryotic wt exuded moderate amounts of the amino acid L-glutamine (Fig. 33).

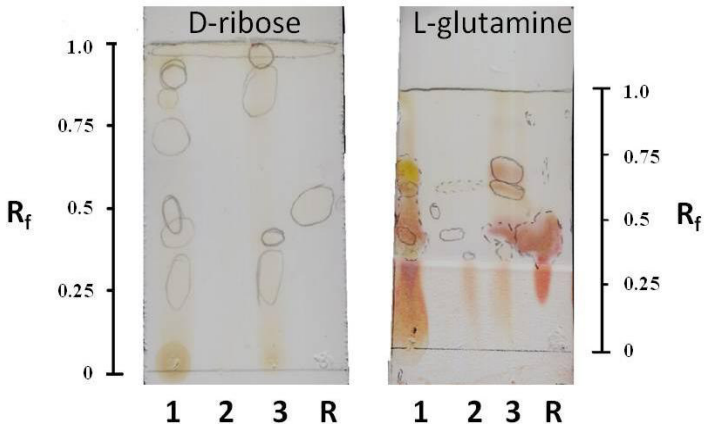

Fig. 33: **Thin layer chromatography of GD of *S. commune* showing D-ribose and L-glutamine. 1** – strain ΔKu8070 T 14(4)xC6 with 0.002 mM azoxystrobin (strobilurin). **2** - strain 12-43x4-39. **3** – strain ΔKu8070 T 14(4)xC6. **R** – reference. (T. Sehrt)

Within a B.Sc. thesis (Sehrt 2013), supervised by myself, a characterization of GD was done. This work focused specifically on the phenomenon of the appearance of GD on mycelia cultures. The different fungi mentioned above were investigated on grounds of their general occurrence of GD and under consideration of several environmental influences and different cultivation conditions at room temperature (RT):

- day light (European day/night rhythm),
- 24 h artificial light,
- addition of 1 mM $PbCl_2$, 0.1 mM $CdCl_2$, 40 mM $SrCl_2$,
- 0.002 mM azoxystrobin

to the culture medium MMNb ½. These different conditions resulted in production of different amounts of GD volumes (Tab. 11). After a short time of 5 d both *S. commune* strains under most treatments produced very high GD volumes in comparison to the ECM fungi.

In order to quantify the elemental content of GD the amount of the heavy metal Pb was tested in additional experiments in 0.5 mM $PbCl_2$. After the cultivation of 1 month, GD were collected. Pb was excreted by all investigated basidiomycete fungi by the respective culture medium (Fig. 34).

Tab. 12: Comparison of Pb contents in 1 mM $PbCl_2$ treated cultures of medium, mycelium, GD of 3 replicates and separate mycelium cultures each. Additionally GD of *P. involutus* are presented in co-cultures with the two ECM fungi *P. tinctorius* and *T. vaccinum*.

Organism	Control - 3 repl. in mg/g	$PbCl_2$ - 3 repl. in mg/g	$PbCl_2$ – 1 fungal culture in mg/g	Substance
Paxillus involutus	0.0002	4.3	1.43	**Medium**
	0.00119	1.1	0.36	**Mycelium**
	0.2	18	6	**GD**
	0.2	25.8	8.6	**GD (+ 2 fungi)**
Pisolithus tinctorius	0.00028	4.2	1.4	**Medium**
	0.00074	0.144	0.048	**Mycelium**
	0.1	11.8	6	**GD**
***Schizophyllum commune* 12-43x4-39**	0.00034	8.96	29.9	**Medium**
	0.00018	0.37	0.013	**Mycelium**
	0.02	495	0.165	**GD**

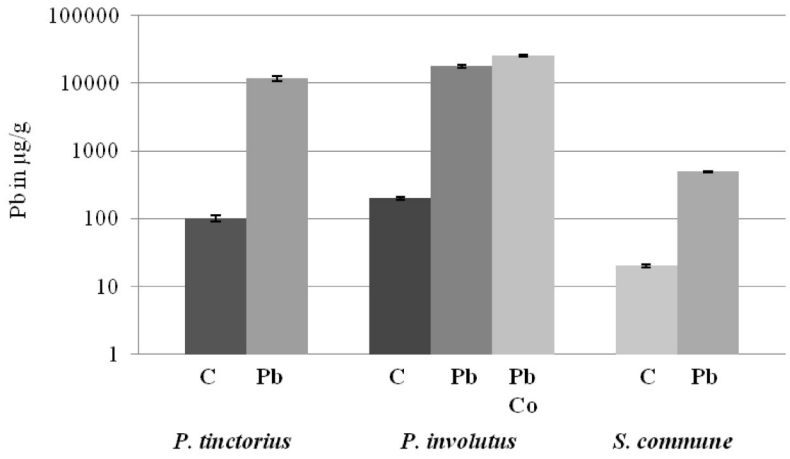

Fig. 34: **Pb content in guttation droplets of *Pisolithus tinctorius*, *Paxillus involutus* and *Schizophyllum commune* 12-43 x 4-39 with and without 1 mM $PbCl_2$ treatment.** The bars present **C** – control on pure MMNb ½ medium, **Pb** – with 1 mM $PbCl_2$ addition and **Pb Co** - *P. involutus* in co-culture with *P. tinctorius* under 1 mM $PbCl_2$ treatment.

Highest amounts of elemental Pb exudation was observed on *P. involutus*: 17.9 mg/g, the control pure MMNb ½ medium was 0.2 mg/g Pb. Even more Pb (25.8 mg/g) was excreted by *P. involutus* in co-culture with *P. tinctorius* and *T. vaccinum*, this is 129 times more Pb content compared to the control. In GD of *P. tinctorius* 11.8 mg/g Pb was measured, while the control contained 0.1 mg/g Pb. This is an increase about 118 times of Pb treatments. So *P. tinctorius* as well as *P. involutus* showed high amounts of Pb in GD in single cultures (Fig. 35). In co-cultures together with two ECM fungi even more Pb exudation was measured. After 1 month, compared to GD content, much less Pb was measured in the medium and mycelium of ECM fungal cultures.

The saprophyte *S. commune* exuded higher amounts of GD though less Pb. Compared to the ECM fungi, *S. commune* did not include that much amounts of Pb of the medium (Fig. 35). So the culture medium contained much higher amounts of Pb compared to the mycelium and GD. Pb treated cultures exuded 0.495 mg/g Pb, the control only 0.02 mg/g Pb, which is 25 times more Pb on *S. commune*. The following Fig. 35 presents the Pb content of the cultivation medium, mycelium and GD under $PbCl_2$ treatment. According to these measurements the *S. commune* contained much lesser amounts of Pb compared to the ECM fungi GD. *P. involutus* included Pb in the mycelium by factor 1000 and exuded Pb by factor 90. The exudation of Pb increased by about 100 times through co-working with two ECM

fungi. The medium with *S. commune* on 1 mM $PbCl_2$ contained 40.000 times higher Pb compared to the control medium.

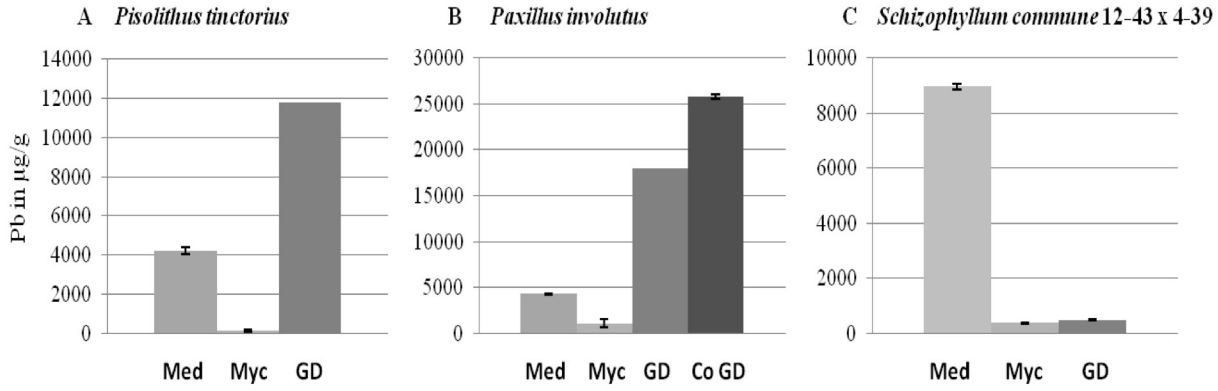

Fig. 35: **Comparison of Pb contents in: Med** - medium, **Myc** – mycelium, **GD** - guttation water of single mycelium cultures and **Co GD** – guttation water of *P. involutus* mycelium co-cultures together with *P. tinctorius* and *T. vaccinum*. The used metal concentration amount was 1 mM $PbCl_2$.

Pb can physiologically replace Ca inside cells. Therefore the amount of Ca in GD was measured. In all fungal cultures the Ca exudation was increased by Pb treatments (Fig. 36). The greatest discrepancy was found in *P. tinctorius*, whereas the least in *S. commune*. GD of *P. tinctorius* under Pb treatment contained increased amounts of Ca (31035 µg/g), more than 3 times higher than the control (8178 µg/g). The exudation of Ca in *P. involutus* nearly doubled under 1 mM $PbCl_2$ treatment (53085 µg/g) compared to the pure control medium (26570 µg/g). Interestingly in GD of *P. involutus* under Pb treatment in co-cultures markedly less amounts of Ca (34034 µg/g) were found. But the exudation of Ca still increased compared to the control, most likely because of the sharing of harmful or non-essential substances and nutrients within the growing medium. *S. commune* under 1 mM $PbCl_2$ treatment exuded around 1.3 times more Ca (19235 µg/g) compared to the control medium (15239 µg/g).

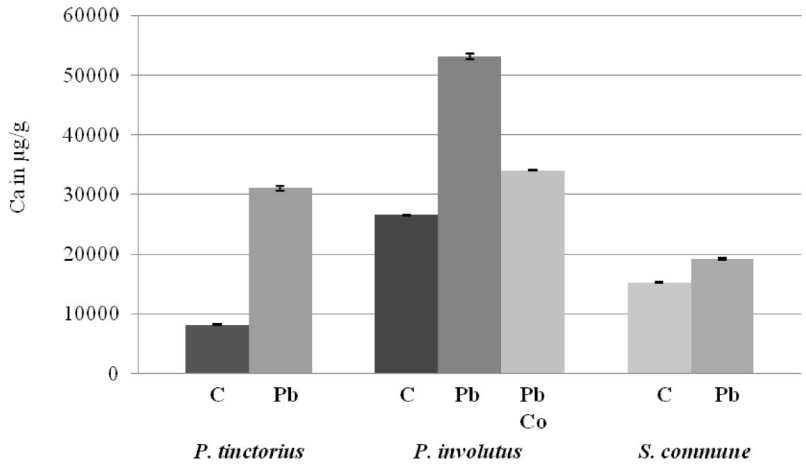

Fig. 36: **Ca content in GD under 1 mM $PbCl_2$ treatment of basidiomycetes *Pisolithus tinctorius*, *Paxillus involutus* and *Schizophyllum commune*. C** – control on pure MMNb ½ medium, **Pb** – with 1 mM $PbCl_2$ addition and **Pb Co** – 1 mM $PbCl_2$ co-culture of *P. involutus* together with *P. tinctorius*.

In order to understand the process of GD production, the water transport mechanism within the fungal cell wall and plasma membrane was tested. Hence the ECM basidiomycetes *P. tinctorius*, *P. involutus* and *T. vaccinum* were investigated by targeting the aquaporins (AQP) localized in the cell membrane being selective water transporter proteins. Elemental Pb exudation was checked under the influence of the AQP inhibitor acetazolamide (AZA) and silver in the form of AgCl and $AgNO_3$. The applied salt concentrations amounted 0.01 mM for AZA and Ag, 0.5 mM and 1 mM for Pb. The GD had been collected for 2 months and were analysed subsequently by ICP-MS/OES measurements.

The early colonizer fungus *P. tinctorius* produced very high amounts of GD (2.3 ml) under influence of AgCl (Tab. 13). Similarly *P. tinctorius* produced high amounts GD (1.1 ml) under AgCl grown together with *P. involutus* (Tab. 13). *P. tinctorius* under influence of 0.5 mM $PbCl_2$ and AZA and in co-culture with both other ECM fungi, early colonizer *P. involutus* and late colonizer *T. vaccinum* exuded a GD volume about 0.7 ml. Whereas the single culture of *P. tinctorius* under influence of 0.5 mM $PbCl_2$ with AZA exuded only around 0.23 ml. Even a greater GD volume was exuded by *P. tinctorius* in co-cultures with *P. involutus* on control plates with MMNb ½ (0.8 ml). The corresponding separate control cultures of *P. tinctorius* exuded 0.28 ml. Under the influence of 0.5 mM $PbCl_2$ in co-cultures with *P. involutus* lower GD amounts (0.5 ml) were exuded compared to the mentioned control co-cultures (0.8 ml). All in all very high GD production was observed under influence of Ag

salt on *P. tinctorius* and the lowest under influence of AZA in co-cultures with two ECM fungi (Tab. 13).

P. involutus exuded high GD amounts on 0.5 mM $PbCl_2$ in co-culture with only one ECM fungus and in control plates. Lower amounts were found in AZA co-cultures with two ECM fungi and in 1 mM $PbCl_2$ and Ag. The highest amounts of GD on *T. vaccinum* cultures were found in control plates, the lowest in the control plates with two ECM fungi (Tab. 13). The results tend to show, that AZA, particularly for *P. tinctorius*, and Ag, particularly for *P. involutus*, affected an inhibition of AQP transported water inside the cell. Possibly a higher exudation of water amounts was induced by GD production. The second early colonizer ECM fungus *P. involutus* produced generally less GD compared to the mentioned *P. tinctorius*. Data show, that *P. involutus* exuded a high GD volume (0.5 ml) under 0.5 mM $PbCl_2$ treatments in co-cultures with *P. tinctorius*. Control cultures produced GD of around only 0.3 ml. Separate *P. involutus* cultures on 1 mM $PbCl_2$ exuded a GD volume of 0.21 ml, whereas co-cultures with AZA of *P. involutus* together with *P. tinctorius* and *T. vaccinum* on 1 mM $PbCl_2$ resulted in decreased GD production (0.095 ml). *P. involutus* in co-culture with AZA together with only one fungus (*P. tinctorius*) and under 1 mM $PbCl_2$ treatment produced very little GD volume (0.05 ml; Tab. 13). The addition of $AgNO_3$ showed no clear connection between co-culture growth and GD production. So separate *P. involutus* cultures with $AgNO_3$ and 0.5 mM $PbCl_2$ and *P. involutus* co-cultures with *P. tinctorius* and *T. vaccinum* on $AgNO_3$ and Pb added resulted in nearly the same production of GD amounts: 0.085 – 0.09 ml.

All in all *P. involutus* produced a high GD volume under $PbCl_2$ and lower under AZA influence. In comparison to the mentioned data moderate GD amounts were exuded by Ag salt and fungal co-cultures. *T. vaccinum* produced in separate control cultures on MMNb ½ only very low amounts of GD (less than 0.07 ml) and under $PbCl_2$, AZA and Ag salt treatments resulted in very decreased or absence of GD (Tab. 13). In consideration of elemental contents of GD several findings appeared. *P. involutus* exuded high amounts of Ag (0.028 µg/g) under influence of 0.5 mM $PbCl_2$ (Fig. 37). These amounts were much higher on co-cultures. So *P. involutus* exuded 0.2 µg/g Ag in coexistence with *P. tinctorius* and *T. vaccinum*. Similarly *P. tinctorius* exuded increased amounts (0.0163 µg/g Ag) in separate cultures without other influences. The guttation of Ag was clearly increased under influence of the ECM fungi *P. involutus* and *T. vaccinum*. But under influence of AZA, the exudation of Ag generally decreased.

Tab. 13: **Amount of GD in ml under influence of 0.5 / 1 mM $PbCl_2$, 0.01 mM AZA and 0.01 mM AgCl / $AgNO_3$. C** – control, **1** or **2 Co** - cultures with one or two fungal partners in one culture. Highest (yellow) and lowest (green) GD amounts of the respective fungus are highlighted. The symbol: / indicates not determined (not evaluable, too low GD amounts).

	P. tinctorius	*P. involutus*	*T. vaccinum*
C	0.28	**0.3**	**0.1**
C + 1Co	0.8	0.02	0.01
0.5 mM Pb + 1Co	0.5	**0.5**	0.07
1 mM Pb	/	0.21	/
0.5 mM Pb + AZA	0.23	/	0.045
0.5 mM Pb + AZA + 2Co	0.7	0.05	/
1 mM Pb + AZA + 1Co	/	0.05	/
AZA	0.28	0.04	0.025
AZA + 1Co	0.025	/	/
AZA + 2 Co	0.005	0.002	/
0.5 mM Pb + Ag	/	0.09	/
0.5 mM Pb + Ag + 1Co	/	0.01	/
1 mM Pb + Ag	/	0.002	/
Ag	**2.3**	/	/
Ag + 1Co	**1.1**	0.01	/
Ag + 2Co	0.38	0.085	/

Pb excretion in GD generally increased under the influence of Ag as well as AZA (Fig. 37). But clear differences occurred in the Pb content depending on the presence of co-cultures. *P. involutus* exuded a very high Pb volume (1.253 µg/g) under Pb treatments. The volume was even increased in co-cultures with *P. tinctorius* and under influence of AZA (1.76 µg/g Pb). So the *P. involutus* exudation of Pb in GD was highest in co-cultures under AZA treatment (Fig. 37).

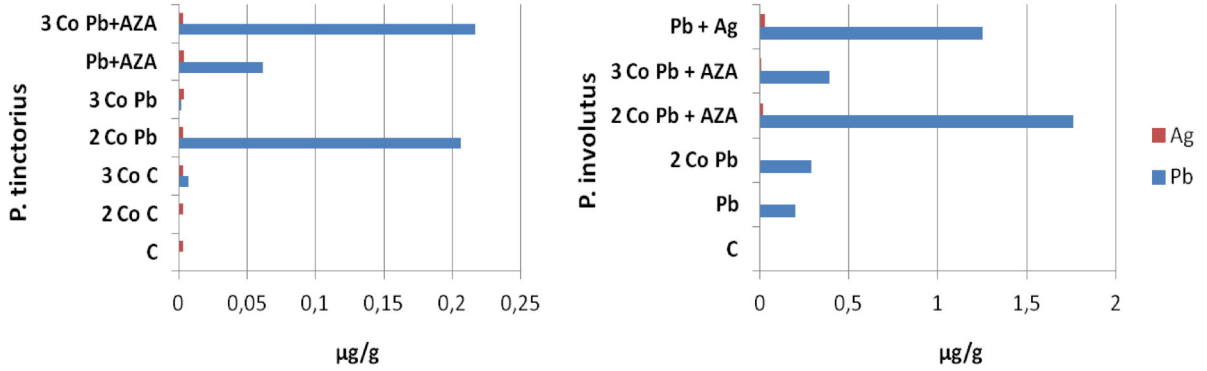

Fig. 37: **Ag and Pb content of GD of the basidiomycetes *P. involutus* and *P. tinctorius* under different treatments. C** – control (separate) culture on MMNb ½, **2/3 Co** – *P. involutus*, *P. tinctorius* and *T. vaccinum* together in co-culture, **Pb** – with 0.5/1 mM $PbCl_2$, **AZA** – with 0.01 mM acetazolamide and **Ag** – with 0.01 mM AgCl or $AgNO_3$.

The addition of Ag and AZA influenced the excretion of essential elements to certain extents. While the essential elements K, Mg, Mn, Na and P showed decreased amounts of GD under different influences of, e.g., Ag or Pb salt, AZA and co-cultures; S, Cu and Si showed a clear increase of exudation (Fig. 38, 39). The Cu excretion was very high in co-cultures of *P. involutus* together with *P. tinctorius* under 0.5 mM $PbCl_2$ (0.906 µg/g). But AZA and $PbCl_2$ affected high amounts of 0.5 µg/g Cu exudation in *P. involutus* co-cultures with *P. tinctorius* and *T. vaccinum* and in co-cultures on $AgNO_3$ 0.41 µg/g Cu. *P. tinctorius* exuded high amounts in separate cultures under AgCl (0.274 µg/g Cu) and low amounts in co-cultures under influence of 0.5 mM $PbCl_2$ (0.203 µg/g Cu) as well as in co-cultures under AgCl (0.207 µg/g Cu).

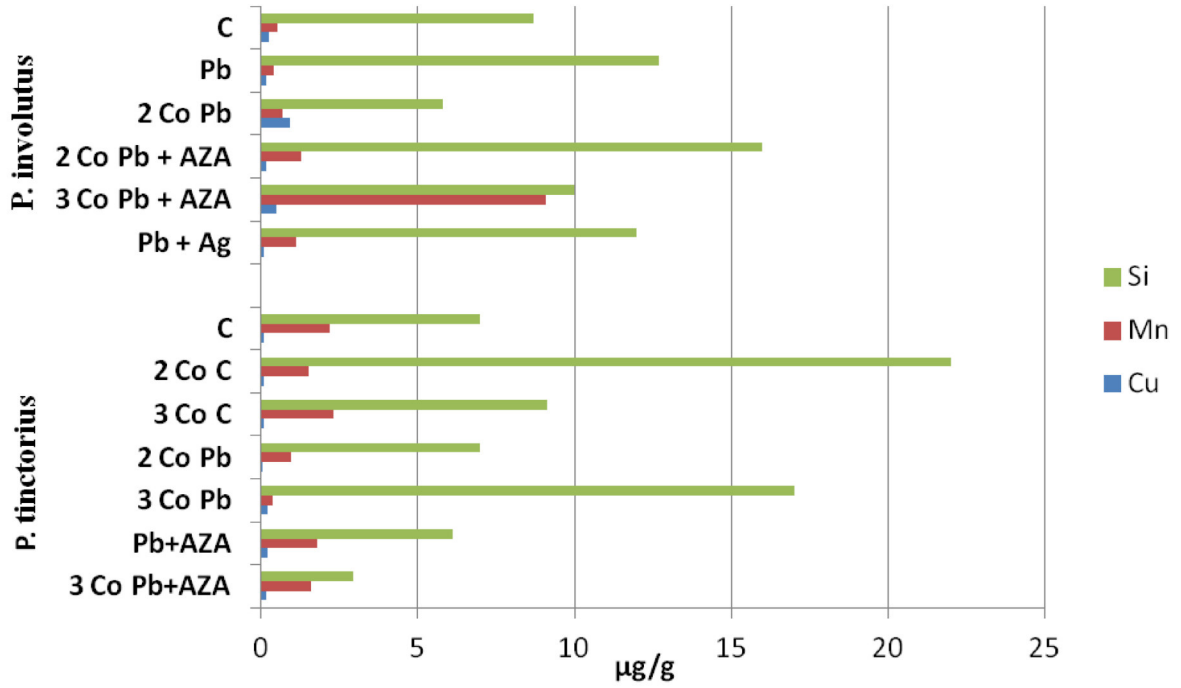

Fig. 38: **Si, Mn and Cu content of GD of the basidiomycetes *P. involutus* and *P. tinctorius* under different treatments. C** – control (separately) culture on MMNb ½, **2/3 Co** – *P. involutus*, *P. tinctorius* and *T. vaccinum* together in co-culture, **Pb** – with 0.5/1 mM $PbCl_2$, **AZA** – with 0.01 mM acetazolamide and **Ag** – with 0.01 mM AgCl or $AgNO_3$.

Ca contents in *P. tinctorius* and *P. involutus* decreased under Pb and increased under Ag and AZA influence. *P. tinctorius* under influence of Ag, especially in co-cultures seemed to be more susceptible to the Ca conduction in GD (32.2 µg/g Ca; Fig. 39). *P. involutus* more intensely responded to AZA and by means of very high exudation of Ca in co-cultures (174.4 µg/g). The exudation of S, especially of *P. tinctorius*, clearly increased (11058 µg/g S; Fig. 39). *P. involutus* co-culture under influence of AZA and *P. tinctorius*, too, exuded high amounts of S (9027.7 µg/g). *P. involutus* under influence of AZA and 1 mM $PbCl_2$, especially in *P. tinctorius* co-cultures, also exuded high amounts (8175 µg/g S). Generally the S excretion was affected the most by AZA in both ECM fungi (Fig. 39).

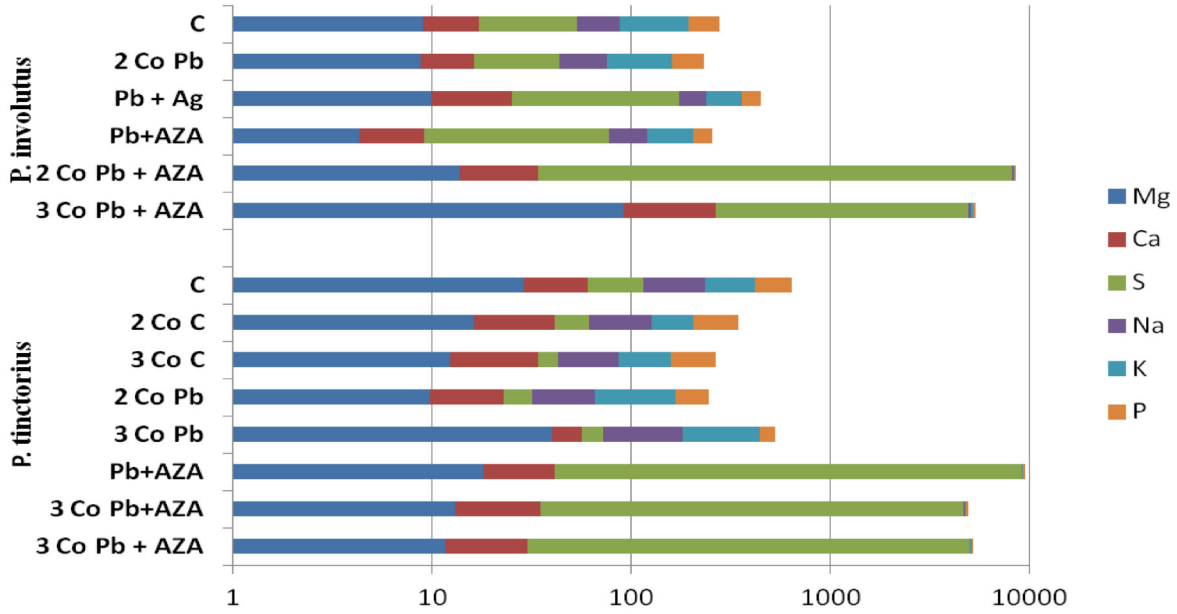

Fig. 39: **Logarithmic scaling of Mg, Ca, S, Na, K and P content of GD of the basidiomycetes *P. involutus* and *P. tinctorius* under different treatments. C** – control (single) culture on MMNb ½, **2/3 Co** – *P. involutus*, *P. tinctorius* and *T. vaccinum* together in one co-culture, **Pb** – with 0.5/1 mM $PbCl_2$, **AZA** – with 0.01 mM acetazolamid and **Ag** – with 0.01 mM AgCl or $AgNO_3$.

Moreover the Pb and Ca content in the condensation water of GD cultures were determinded after 2 month of growth. As well as in *S. commune* wt and mutant strain cultures, high amounts of elemental Pb was measured in the condensation water of the GD cultures (Fig. A1, A2 appendix). The condensation water of wt cultures on 0.5 mM $PbCl_2$ treatments contained only little more Pb (23 µg/g Pb) compared to the wt control (20 µg/g Pb). But condensation water of wt cultures on 1 mM $PbCl_2$ contained clearly more (46 µg/g Pb). High Pb values were determined in mutant cultures on 1 mM $PbCl_2$ treatments with 60% increase compared to the wt (Fig. A1).

Ca contents showed clearly other results in the condensation water of *S. commune* cultures (Fig. A2). In wt cultures high volume (more than 4000 µg/g Ca) was measured in the control and low values (less than 2000 µg/g Ca) in 0.5 mM $PbCl_2$ treatments. In the case of the *S. commune* mutant opposite results were detected. High Ca values of more than 2000 µg/g were measured in the control cultures, but very high amounts (more than 5000 µg/g) were found under 1 mM $PbCl_2$ treatments. So *S. commune* tends to emit non-essential or toxic elements like Pb into the fungal culture atmosphere, while Ca in most cases showed a clear decrease under $PbCl_2$ treatment compared to control cultures.

4. Discussion

4.1. Characterization of ectomycorrhiza in mesocosms

The inoculation of mesocosm systems with mycelium of the early colonizer *Pisolithus tinctorius* and *Paxillus involutus* as well as the late colonizer *Tricholoma vaccinum* was not successful. After the experimental period of 1.5 years no short roots or fungal structures (extramatrical mycelium and hyphal mantle formation) of the three fungi could be determined according to Agerer (2001). Instead short roots of the ascomycete *Cenococcum geophilum* were found. This ascomycete fungus without a formation of fungal fruiting bodies can be described as "multi" colonizer with little plant host restriction (Smith and Read 2008). Furthermore *C. geophilum* is well known for its worldwide geographic distribution in connection with wide ranges of plant partners (Molina *et al*. 1992). Moreover this ascomycete was the dominant species in the mesocosm pots, which was already described from other experimental setups by Koide *et al*. (2005).

C. geophilum did not seem to be very much influenced by strong moisture or even temporary water logging. With its enormous resistance for drought (Coleman *et al*. 1989, Molina *et al*. 1992) this fungus is very suitable for harsh, clayey and disturbed substrates which was typical for the heap material. Additionally it is described, that its abundance increases under dry conditions (Pigott 1982) which was probably also induced by the weakening of the other ECM fungi in the substrate. So all in all the apparent self infection of *Picea abies* and *Pinus sylvestris* by *C. geophilum* within the mesocosm experiments seems to improve the plant water relation like it is described by Mikola (1948) and Pigott (1982). Therefore it preserves the ECM system in soil contexts like in the mesocosm experimental data shown. In the case of the given soil type conditions, the mobile soil metal concentrations did not seem to influence the occurrence of *C. geophilum* negatively.

For molecular characterizations of ECM fungal material, especially short roots, the ITS region of the rDNA gene cluster with the 5.8S rDNA gene, ITS1 and ITS4 were used. Since ECM fungi do not form a monophyletic group no ECM specific PCR primers do exist. Therefore generally primers with high specificity for basidiomycetes and ascomycetes with target ITS regions are chosen. The rDNA gene clusters are present in about one to one hundred copies per each genome in the fungal cell nucleus which provide a suitable target region for genetic analyses (Landweert *et al*. 2002). The ITS region has a size between 600 and 800 bp. It contains two variable, non-coding regions localized between the highly

conserved little 18S and big 28S subunit of the rRNA genes. The whole sequence surrounds the functional 5.8S rDNA (Gardes und Bruns 1993). In the same way *C. geophilum* could be detected. So its existence in the mesocosms was proved.

The lack of fungal short roots of *P. tinctorius*, *P. involutus* and *T. vaccinum* might have originated from low growth rates and lower tolerance in regard to low water capacities and dry soil conditions. According to Agerer's (2001) exploration types of immense functional relevance *P. tinctorius* and *P. involutus* are long distance ECM with characteristically smooth short roots and few but very differentiated rhizomorphs. *T. vaccinum* belongs to the medium distance ECM that forms rhizomorphs, too. Both types seem to be adapted to generate nutrients from far distances and access broad fields. In contrast *C. geophilum*, a short distance exploration type with voluminous amounts of emanating hyphae, that forms no rhizomorphs, seems to rely on nutrients in its direct surrounding and is well adapted for this. These facts give *C. geophilum* advantages especially in surviving in adverse substrates.

4.2. Concentration of elements in ectomycorrhiza

An individual amount of element content occurred depending on species, as well as biotic and abiotic factors in ECM fruiting bodies. BCF values from collected *P. tinctorius* and *P. involutus* fruiting bodies, originated from a former uranium mining heap, showed unexceptional BCF values > 1. This indicates clearly accumulation of the elements into fungal tissue and reduced amounts in the growth substrate. (Bahadir *et al*. 1995). Emanating of these BCF values, the early colonizer fungus *P. tinctorius* accumulated the elements Cs (BCF 12), Pb (BCF 40), Sr (BCF 11) and U (BCF 28) in evidence to high BCF values. But the other investigated elements Cd (BCF 7) and Ni (BCF 9) showed high BCF values too. Therefore *P. tinctorius* is appropriated for remediation approaches, especially over longer periods. Dense colonization and the preference for heap areas and post-mining landscapes (Kim 2002), amplifies its aptitude for harsh, acidic, nutrient poor substrate and unskilled areas (Krieglsteiner 2000, Dörfelt and Bresinsky 2008). *P. tinctorius* as early colonizer fungus appertains well for pioneer succession regions, even in larger scales, due to its ability of unspecific colonization of pioneer trees (Smith and Read 2008; Last *et al*. 1983, 1987). Turnau *et al*. (1994) and Galli *et al*. (1993) described for *P. tinctorius* a revealed accumulation of Cd in the cell wall, especially in outer pigmented layers. While Galli *et al*. (1994) described heavy metal binding to cell wall components such as chitin, cellulose

derivatives and melanin. So the benefit of the ECM fungal association is mainly connected to the potential prevention of translocation of heavy metals into the host plant (Khan *et al*. 2000).

But the limit of the heavy metal concentration in the plant host is not always ensured. So no decreased Cd concentration was found in the ECM symbiosis *P. abies–P. involutus* (Godbold *et al*. 1998). But fruiting bodies of *P. involutus* showed in this experimental setup very high accumulation values for Cs (BCF 66). The other investigated elements Cd (BCF 2), Ni (BCF 3), Pb (BCF 2), Sr (BCF 9) and U (BCF 5) still showed accumulation values, albeit in lower ranges. Blaudez *et al*. (2000) confirmed binding of Cd onto cell walls, with dependency on the membrane potential, beeing a essential metal-detoxification mechanism resulting in the accumulation into vacuolar compartments. *P. involutus*, as early colonizer with unspecific settlement of trees, showed a preference for Cs accumulation, which appertains it for remediation applications too. Fruiting body distribution of this species did not appeared in high numbers, like *P. tinctorius*, but still showed a widespread occurrence.

Both early colonizer fungi appear to dispose over essential cell detoxification and therewith protection strategies. After Haselwandter and Berreck (1994) both early colonizer ECM fungi were well appertained for unsettled rarely developed substrate and accumulate heavy metals and radionuclides in high ranges. Heinrich (1992) described especially for *P. involutus* 21.4 Bq/kg dry weight ^{137}Cs. Whereby the uptake of radioactive Cs is mainly substrate specific (Eckl *et al*. 1986). So the Cs transfer from soil to fungi correlates with the pH. The solubility and mobility of Cs increases with decreasing pH. Clay-Cs^+ bounds exchange by H^+, but with increasing pH less ion exchange results. In consequence also Cs remains bounds and is therefore not available for fungi furthermore (Scheffer and Schachtschabel 2010). Oolbekkink and Kuyper (1989) described ECM fungi more efficient in radionuclide accumulation than saprophytic fungi. Dighton *et al*. (1991) showed 40% increase of radionuclide immobilization, bound by hyphae. So the fungal biomass in the soil could immobilize substantial quantities of especially radiocesium for a unknown period of time (Haselwandter and Berreck 1994). With regard to variances in BCF values for Cs in the two ECM *P. tinctorius* and *P. involutus* also Klan *et al*. (1988) described a range between four orders of magnitude of BCF`s for Cs transfer from the soil into hyphal fruiting bodies. In studies of Clint *et al*. (1991) short-term influx of ^{137}Cs into fungal hyphae ranged from BCF 85 for *Cenococcum geophilum* to 276 for *Mycena polygramma*. These facts predestine both ECM basidiomycetes *P. tinctorius* and *P. involutus* as objects for remediation approaches. Living and dead microbial cells are capable for radionuclide accumulation, due to the fact that

radionuclides bind to extracellular polysaccharides of the cell wall, which is metabolism independent. In principle it can be described as biosorption process.

Looking in general into the microbial soil, for *S. cerevisiae* a specific extrusion pump, presumably a Ca^{2+}pump was described. This pump enables cells to regulate the accumulation of, amongst others, Sr^{2+} to different levels. (Theuvenet *et al*. 1986). Next to active membrane pumps, substrates of the secondary metabolism are described as radionuclide scavenger. So for the basidiomycetes fungi *X. badius*, *B. erythropus* and *B. mirabilis* the cap pigments badione A and norbadione A, pulvic acid derivates, seems to be responsible for radiocesium accumulations (Aumann *et al*. 1989). Therefore a large species-specific difference in heavy metal and radionuclide accumulation of fruiting bodies of basidiomycetes exists.

4.3. Heap substrate and influence of ECM on its metal distribution

The heap material of the former uranium mining heap nearby Ronneburg, Thuringia, Germany seems to show processes of exhumation after the experimental period of 1.5 years. This resulted in released of larger values of elements compared to the origin substrate, likewise described by Mirgorodsky (2014). While the cultivation of the pots, high amounts of fine particles within the soil solution were probably mobilized within the watering. Thereupon an increase of bioavailable particles, especially elements, proceeded (personal comment of Prof. Dr. G. Büchel, Friedrich Schiller University Jena). Another element release supporting process in the experimental mesocosm system could be the release of root exudates, which results over a longer time in multiplied bioavailability of elements. This shows temporarily mobilization and enrichment of elements. Indeed the pH did not decreased over the experimental period within the heap material, but increased in both substrates. This tends to be contradictory. The element availability varied under changing pH. The non-essential elements Cd and Zn were well available in pH ranges lower 6.5. As well as Ni and Pb, which were well available in lower pH ranges of 5.5. The availability of the essential and the trace elements decreased especially for Mn and increased especially for Fe in low pH ranges, while the availability of Ca and Mg increased by rising pH. Accordingly the toxicity of the soil solution depends on the pH (Scheffer and Schachtschabel 2008). A minimal heavy metal and radionuclide availability exists under neutral pH ranges between 6.5-7.5 (Roempp 2006). Under pH conditions of 3-4, like from heap material, higher availabilities of heavy metals and radionuclides were expected. By comparing the substrate loess loam with pH values of 6.5-7.5, lower heavy metal and radionuclide availabilities were expected.

Homogenization and compaction of the substrate during filling into the pots is important for element availability too. As a consequence thereof air introduction could favor oxidative processes. A mixing of the substrate created new soil particle surfaces on which more easy erosive processes can take place. The more new particle surfaces exists, the more release of elements can take place.

Magnesium sulphates epsomit ($Mg(SO_4)*7H_2O$) and hexahydrite ($Mg(SO_4)*6H_2O$) occurrence *via* crystal formation appeared at surfaces of the heap material (Fig. 13). Fibrous aggregates, efflorescence and crusts are typical states of appearance. Epsomit, the same as its dehydrated version hexahydrite, easily dissolves in H_2O and is very sensitive against air movement like wind. Usually epsomit and hexahydrite S-minerals occur as by-product at the processing of pot ash salts (Hawthorne *et al*. 2000). Hexahydrite is chemical similar to epsomit. It is unstable, emits crystal water in dry conditions and melts under high humidity. Through dehydration from epsomit, hexahydrite as secondary mineral is formed (Rösler 1987). Both S-minerals are build in consequence of acid mine drainage processes. XRD investigations showed, that epsomit with proportionally 73% forms the major mineral portion of the heap material. Nearly all other components were S-minerals too. Hexahydrite forms the second frequent mineral (14%) followed by 8% alunogenum ($((Al(H_2O)_6)_2(SO_4)_3(H_2O)_5)$, 3% gypsum ($CaSO_4*4H_2O$) and 2% others. High S and Al amounts reflected the elemental composition of the heap substrate too. But in comparison to the loess loam substrate the low pH ranges of 3-4 in the heap material influenced the bioavailability of these elements.

Delicate mats of algae occurred in all pots after 1.5 years and the attempted plants were very sensitive for dehydration and backwater, which appeared *via* stunted and crippled growth to the point of death of the plants. Growth conditions of the heap material like tendencies for silting up, very less amount of carbonate and very little organic, complicated the growth additionally. *P. sylvestris* showed thereby best abilities for adaptation and establishing under these circumstances with and without ECM fungi, whereby ECM inoculations considerably extended their lifetime. *P. abies* showed very less adaptation to the substrate conditions and reacted very sensitive to given soil settings, more or less independently of ECM fungal inoculations. The planned application of *B. pendula* was quit after first germination trials due to less success during germination in pre-cultures, caused by the mentioned substrate conditions.

Element contribution in heap material after the experimental treatment showed various distribution pattern. The fungal occurrence can change mobile metal concentrations and therewith their bioavailability. The essential element K in pots with ECM fungal inoculation,

showed increased bioavailability in the roots, influenced the upper soil and was less available in root indirectly influenced deeper soil zones. More increased availability was found in upper soil zones of pots without ECM fungi and only with plants. Less K was available in the deeper soil zone in pots with ECM the same as without ECM, but with plants. In control pots without any vegetation, higher K amounts were available in deeper soil zones compared to decreased K amounts in upper root influenced soil zones. Generally K seemed to be more available under influence of ECM fungi. In all other pot treatments, including the control pot, elemental K was less available. The distribution pattern of K tends to resembles ones of Mn and Ca. Elemental Ca was generous bioavailable in both soil depth. Higher Ca was bioavailable in the pots, within both depth, with inoculated ECM fungi. But without ECM fungi and only with plants, Ca availability in the deeper soil zones of 50 cm was increased compared to decreased availability in upper soil zones of 3 cm. Ca availability in control pots were not substantial different from the element content of the original heap material. Generally the Ca contribution pattern conducts similar to Sr. All in all it seems like Ca availability was most influenced by ECM inoculations resulting in higher bioavailability in both soil depth with influence of ECM fungal inoculation. Elemental Mg availability was decreased under ECM fungal inoculation. So pots with ECM showed in upper root influenced soil regions decrease and in deeper soil regions increase of Mg. Compared to pots without ECM but with plants, elemental Mg was minimal more available compared to pots with ECM and much higher available in deeper soil zones. In control pots without any growth, higher Mg amounts are measured, compared to the other pot treatments, and little less in deeper soil zones. Mg can be determined or consumed by ECM in tendency. The distribution pattern of Mg resembles S. Elemental distribution of Mn showed increased availability in upper soil regions. In upper soil regions under ECM supply, high elemental Mn occurred, while in deeper regions clearly less amounts are measured. Control pots showed minimal reduced amounts in this area compared to pots with ECM, but clearly decrease in deeper soil regions. Low Mn amounts are found in pots without ECM but with plant growth in upper root influenced soil regions. Still low amounts occurred in deeper root indirectly influenced soil regions, but still more than in pots with ECM with similar values compared to the control. Mn was rarely influenced by ECM. S showed increased elemental amounts in upper soil regions of control pots without growth. In deeper soil zones low amounts were measured, compared to the upper soil regions and again low amounts compared to treatments with only plants and without ECM. Pots with ECM showed in general low amounts of S, but similar amounts in

both soil zones. Pots without ECM and with plants showed nearly same amounts in both soil depth. Again S seems to be rarely influenced by ECM or plants.

The non-essential elements showed again various distributions. Little higher Cd availability compared to the deeper soil region were found in the upper soil region under influence of ECM fungal inoculation. A minor tendency of Cd availability could be registered in pots with plants and without ECM, in the upper root influenced soil region. In control pots Cd was little higher available in the deeper soil region. Cd showed generally no clear difference of availability under the several treatments control, with and without ECM, and with plants.

Cs showed lowest availability under ECM fungal addition in both soil regions. In control pots without any fungal inoculation or growth high amounts of Cs were measured. Pots without ECM but with plant addition showed around $1/5^{th}$ increased availability of Cs. These results tends to resume that with ECM fungal addition, 32% lesser Cs was available in the substrate and therewith potentially determined or at least bound by the help of the ECM. This accompanies with high BCFs for ECM fungi *P. tinctorius* (BCF 12) and *P. involutus* (BCF 66). Additionally the contribution pattern of elemental Cs resembles clearly these of Al.

Elemental Pb availability in the heap substrate was decreased in control pots within both depth, with very low amounts in the deeper soil regions. Pots with ECM showed 1/6 higher amounts of Pb in upper root influenced regions compared to the deeper part. Pb availability from pots without ECM and with plants showed no clear difference in availability compared to ECM fungal addition. Generally low amounts of elemental Pb was available in deeper soil zones, but ECM or plant addition seemed to increase Pb availability equally.

The availability of elemental Al was increased ($1/4^{th}$ to $2/4^{th}$) in control pots within both soil depth without any fungal influence or growth. Low Al availability was detected in pots with ECM addition, nearly $2/4^{th}$ reduced. Pots without ECM but with plant growth showed $1/4^{th}$ more Al availability compared to pots with ECM and $1/4^{th}$ decrease compared to control pots. Due to the fact of low elemental Al availability under ECM influence, Al seems to be bound and determined under ECM fungal influence. In case of Sr most proportion was available in inoculated pots, with tendency to increase in the upper soil zones of the root space. Less Sr amounts were bioavailable in pots without ECM inoculation and with only plants. Whereat plants seemed to influence the Sr availability too. Only $1/7^{th}$ less Sr availability was measured in pots without ECM but with plant growth. Low amounts of Sr were measured in control pots, $2/7^{th}$ decreased. So Sr availability seems to increase under ECM and plant influence.

The availability of U was not very different in several pot treatments. High availabilities were found in upper soil zones with ECM addition. In contrary in deeper soil zones low U amounts were found. Control pots showed in the upper soil region high U availability compared to deeper regions, but still little less compared to pots with ECM addition. Pots without ECM but with plant growth, showed low element availability in upper regions and increased in deeper soil zones. So generally the tendency was found, that the elemental U availability was increased in the upper soil zones and little low in deeper regions, but only rarely influenced by ECM or plant addition. Only a very little higher availability was found in the upper root influenced soil region under ECM influence.

The percentage ratios of non-essential and essential elements of heap material was additionally compared. In not overgrown control pots the portion of non-essential elements of Cs:Sr:U was 35:25:40, whereas this portion differed clearly in pots with ECM (22:36:42). So with ECM addition the bioavailable Cs and U proportion seems to decrease, while Sr values were increased. This tendency was seen in pots without ECM but with plant addition 29:33:38 (Cs:Sr:U) too, indeed much more pronounced with ECM addition. The percentage ratio of essential elements in not overgrown control pots showed the range 24:6:30:40 for Ca:K:Mg:S, while this distribution changed under ECM influence (30:6:30:34). So more Ca and less S were bioavailable but the same proportions of K and Mg under ECM influence. The distribution of essential elements in pots without ECM but with plant addition considerably resembles control pots with 24:6:32:38 (Ca:K:Mg:S). Exemplifying these percentage contributions of elements, the influence of ECM in heap material was seen, even without visible ECM structures.

Statistical analysis of heap material soil data resulted in significant differences in control pots in between two depths for S and U. For pots with ECM addition significant difference was found for the elements K, Mg, Mn and U. Data from pots without ECM, but with plants, no significant elemental difference was found in between two soil depth.

4.4. Influence of ECM on metal distribution of loess loam substrate

The loess loam material, with different soil structure characteristics compared to the heap material, showed less processes of exhumation. Most of the investigated non-essential and essential elements Cd, Cs, Ni, Pb, U, Mg and S increased over the experimental period. Whereas the element amount of Sr, Ca, K and Mn decreased, whereby the distribution pattern of Sr and Ca proceed similarly. As well here the release of root exudates could have been

contributed the increased bioavailability of the elements. The advantage of this soil substrate was fast and uncomplicated settlement of plant colonizers either *via* wind entry or from autochthonous soil seed banks. After 1.5 years experimental period, all pots were well overgrown with annual or biennial herbs. And again the element data of the loess loam showed after the experimental treatments control, with ECM and without ECM but with plants minimal differences in between.

Values of non-essential elements mainly decreased. Cd in pots with ECM minimal decreased in the upper and deeper soil region compared to the control. The Cd amounts did not vary in between the two soil depth. In control pots, no change of Cd values were detected in between the two soil depth 3 and 50 cm, but more elemental Cd was bioavailable in upper root influenced soil regions compared to the treatments with ECM and without ECM, but with plants. Pots without ECM but with plant addition showed minimal and in comparison low Cd availability in upper soil zones. Thereby most elemental Cd values were detected in the deeper soil regions. Cd availability seemed to be generally reduced, but differences in between the treatments were not very pronounced. Whereas ECM additions tended to decrease Cd availability in this loess sand substrate.

Cs availability in control pots showed decrease in upper root influenced regions and increase around 2/6 in deeper zones. Pots with ECM addition showed in upper soil regions same availabilities for Cs like pots without ECM but with plants and in deeper soil regions same availability like the control. Cs availability was in deeper zones little increased under ECM influence (around 10%). Pots without ECM but with plants showed clear decrease of Cs amounts in deeper regions. Cs availability seems to be moderately up to little influenced by ECM addition via value increase, but more strong influenced by plant addition.

Ni availability was not too much different in between the pot treatments too. So in control pots high Ni values were determined in upper root influenced soil regions and low in deeper regions. Pots with ECM addition showed Ni decrease compared to the control, but increase in the upper region compared to ECM absence. In the deeper region high Ni values occurred under ECM addition compared to the control, but minimal lower values compared to pots without ECM. In pots without ECM, but with plants in upper regions low and in deeper regions high Ni values were found. All in all it seems like Ni amounts increase or accumulate in deeper soil region, but this process can be influenced by ECM or/and plant addition.

Pb distribution was clearly most decreased under ECM influence in both soil depths. While Pb amounts in control pots were little lower, in deeper regions high amounts were detected. Absence of ECM but with plant addition influenced Pb availability in both depths

rarely. So generally it seems, that Pb availability was clearly influenced by ECM addition. It even decreased around 1/6th in deeper soil regions compared to the control. This fact matches obviously to high amounts of exuded Pb values from GD of ECM fungal mycelium and therewith reduction in the substrates but the same time preventing fungal cell toxification.

Sr availability was decreased in upper soil regions and increased in the deeper. So ECM addition mostly increased the Sr availability in upper root influenced regions, but decreased in deeper zones around 1/7th compared to the control. Treatments with only plants showed low Sr values in the upper and deeper soil region, in deeper zones even around 2/7th reduction compared to the control. These data link to the implications, that ECM and plant addition influences the substrate this way, that present Sr amounts are balanced in the soil-organism system.

The U availability was generally increased in deeper soil regions and less different in the several treatments in upper soil regions. In the control U was nearly similar available in upper and deeper zones, but clearly lower values were detected compared to pots with ECM addition. So the pots with ECM showed high bioavailable U values in both soil depths. Pots without ECM, but with plants showed low U values in the upper region, but increased values in the deeper. All in all, in deeper soil regions ECM addition influences the U availability around 1/4th compared to the control.

In case of the essential elements the process of exhumation appeared more clear. Ca availability differed in the control between the two soil depth about 1/8th, with increased amounts in deeper soil regions. While Ca values of pots with ECM differed negligible between both depths, high values were found in the upper soil region compared to the other treatments. In pots without ECM, but with plants, high values were found in the upper root influenced soil regions. But these amounts were still lower compared to ECM pots. Generally Ca bioavailability was negligible influenced by ECM addition, but ECM addition balanced Ca amounts in comparison to control soil.

High K was found in control pots of upper soil regions compared to treatments with and without ECM and plant with 1/7th lower values. In deeper soil regions K was 2/7th decreased. Elemental K values decreased within ECM addition in upper regions compared to the control and decreased rarely more in deeper soil regions. In the upper soil region, pot values of only plant treatments differed not from pots with ECM addition. Only tendencies were found for increase of K in upper and decrease in deeper soil regions in comparison to pots with ECM. So it seemed that K is clearly influenced by ECM and plant addition, especially in the upper root influenced soil regions.

Mg availability showed very rare differences in between several pot treatments. Pots with ECM addition showed no change in Mg content in between the different depth, but was clearly reduced, around 3/7th, over the experimental period of 1.5 years. Control pots showed decrease in upper and in deeper soil regions. Pots without ECM, but with plants showed the opposite, increase in upper and decrease in deeper soil regions. All in all it seemed, like Mg was not considerably influenced by ECM.

Elemental Mn availability showed more variation. So control pots showed constant low values in between both soil depth, with lower amounts in upper root influenced regions and little higher amounts in deeper soil regions compared to pots with ECM addition. In pots without ECM, but with plants, low available values were measured, with high amounts in upper and lower amounts in deeper soil regions. In general the Mn bioavailability was higher after 1.5 years of experimental period with increase of availability in upper soil regions, especially under ECM influence, and decrease in deeper soil regions.

S showed clear decrease of values in both soil depth after 1.5 years. Generally low values in upper root influenced soil regions and clearly high values in deeper soil regions were found. So in upper soil regions low amounts in the same range were found for control and ECM pots, in comparison to deeper soil regions with more than 2/4th increase of S values. Clearly decreased bioavailable S amounts were found in pots without ECM, but with plants in upper and deeper soil regions. These results were inhomogenous cause same increase and decrease values were found for control and ECM pots. This could guide to conclude, that ECM addition determines S like clay minerals or other soil structures do.

The percentage ratio of element distribution in loess loam showed clearly a very different picture compared to the one of heap material. The non-essential element distribution in control pots for Cs:Sr:U was 0:95:5. This ratio did not differ in pots with or without ECM, but with plants. Only in deeper soil regions the elemental distribution varies after the range 0:90:10 (Cs:Sr:U) in pots with ECM and plant addition. The essential element distribution for Ca:K:Mg:S followed the range 85:4:15:6 in control pots of upper soil regions. With ECM addition the ratio varies in upper soil regions after the range 88:1:8:3 (Ca:K:Mg:S) and in deeper soil regions 82:1:6:5 (Ca:K:Mg:S). Percentage distribution of essential elements in pots without ECM, but with plant addition showed low S amounts in the elemental range 82:2:14:2 (Ca:K:Mg:S) in upper and 80:2:13:5 (Ca:K:Mg:S) in deeper soil regions. Main proportion in loess loam sand generates Ca followed by Mg. Both seemed to be influenced by ECM addition. In upper regions with increased amounts and in deeper regions with decrease. S and K represent a minority in the loess loam sand and especially S was clearly influenced

by ECM addition with low amounts in the upper root influenced region and high amounts in the deeper regions. K does not showed much intense influence by ECM addition.

Statistic analysis resulted in significant differences in control pots in between two depths for Ca, K and Mg as well as for Sr. Statistically no difference was found in pots with ECM addition. This tends to the resume, that ECM even in indirectly influenced soil regions have an positive effect. Statistic data from pots without ECM, but with plants, showed significance for non-essential elements Cd and Ni. The question about changes of the element content of soil in defined soil microstructure within mesocosm experiments about 1.5 years by the help of ECM could only unsatisfactory become answered. By comparing before and after planting and inoculation with ECM fungi the data show, that the element change was minimal in the bioavailable fractions I and II, analyzed after the protocol of Zeien and Brümmer (1989). So possibly there is an answer to find focusing the analyzing method as well the experimental setup.

The sequential extraction after Zeien and Brümmer, with single investigation of each fraction by decline of pH and therewith analyzing of more bound elements, could be compared to other extraction methods like the one of Förstner and Calmano, 1982. In this protocol fractions I-III are summarized to one extraction step and therewith the extraction pH amounts constantly at 6 for the bioavailable, the deliverable and as well the Mn bound fraction. Rising pH follows only in the additional fractions. However in the method of Zeien and Brümmer fraction I starts with pH 4.76 and rises in the following fractions II and III up to 6, again with decreasing pH from fraction IV on. An starting pH with consequent decrease in pH could maybe yield in more consistent binding results of the elements.

4.5. Effects of metals on plant growth

In both substrates the inoculated ECM fungi *P. tinctorius*, *P. involutus* and *T. vaccinum* have not established an ECM symbiosis with visible short root formation. But nevertheless different effects occurred in pots with initial ECM inoculation.

Root length of the trees required vitality and establishment data of the ECM fungal symbiosis partners. So root length data from *P. abies* and *P. sylvestris* differed clearly. The longer primary roots of *P. sylvestris* showed 7 cm difference compared to those of *P. abies*. Secondary roots differed among 8-9 cm between the two species. But in both cases the addition of ECM fungi resulted in 20% increase of primary root length, 17% for *P. sylvestris* and 22% for *P. abies*. Secondary roots were not affected intensely as well. So secondary root

length data showed increase, in lower values, of 14% for *P. abies* and 15% for *P. sylvestris*. Whereby secondary roots are preferred for the short root formation. Generally root length data from both trees showed increase and therewith enriched possibilities for better availability of elements, especially nutrients as well as water, from the substrate. By comparing branches of both trees it was found, that the ECM inoculation influences positively too. So more branches occurred on mycorrhized roots of *P. abies* and *P. sylvestris*. For *P. abies* 29% and for *P. sylvestris* 13% more branches with ECM inoculation compared to single tree roots. These facts confirm increased organic material production for mycorrhized trees even without visible short root formation or external ECM entry under these heap substrate conditions. Harley and Smith (2008) as well as Moser and Haselwandter (1983) described for mycorrhizal infection generally significant effects on mineral nutrition of plants. So even presence of ECM can promote amelioration of soil toxicants by extrahyphal slime for example (Tam 1995). Khan *et al*. (2000) focused under these soil–ECM establishment circumstances two main issues for further benefits. So firstly fungal types decide pronounced about mycorrhizal roots formation and therewith extramatrical biomass production. This can determine possibilities for environmental amelioration for different organisms as well succession of ecosystems and moreover the soil conditions including nutrients, minerals, water, pH and soil structure. So for several soil conditions different organisms are suitable. Climate, abiotic soil structure, origin of fungal and plant organisms as well as the supply of nutrients influence the soil-ecosystem development by increase or decrease effects.

4.6. GST activity in fungal cultures and ECM

Generally ECM relationships, between fungus and tree, can improve plant establishment and growth clearly. It can ensure amelioration of heavy metal and radionuclide toxicity for the organisms and their environment. GST`s therewith provide cytosolic protection against toxic imbalances of the cell, as one type of different detoxification options of the cell.

With the hypothesis, that fungi, as well as plants, would show increased GST activities, when grown on element loaded sites, cytosolic GST tests were performed. It should become proven if the fungal metal tolerance involves GST activity. Substrate contamination was simulated by the addition of heavy metal and radionuclide salts in growth media.

GST activity for Ni and Sr, and low for Cs occurred from both early colonizer fungi, while the BCFs differed clearly, with increased bioconcentration ability for Ni of *P.*

tinctorius. But interesting is the finding, that the GST activity decreased about 3 times in the early colonizer fungal tissue under presence and influence of another fungus. Thus *P. involutus* well appertains for remediation approaches, especially on fields with high radionuclide loads of Cs and Sr. GST activity values of symbiosis of *P. involutus* with young *P. sylvestris* trees from germination medium experiments with 0.1 mM $CdCl_2$ showed in this early stage while the germination, already protection by increase in roots and decrease in C+S. Compared to GST activity values of *P. sylvestris* grown on 1 mM $PbCl_2$, these similar results showed very low values and only tendencies without the fungal partner.

In comparison to the early colonizer fungi, the GST activity of the late colonizer fungus *T. vaccinum* was relatively low. Interesting was the point, that *T. vaccinum* in fungal co-cultures together with *P. tinctorius* reacted on 1 mM $PbCl_2$ with increased GST activities (2.7 µmol/g/min) compared to single *T. vaccinum* fungal cultures on the same metal salt (2 µmol/g/min). So the presence of fungal partners seems to increase the GST activity in fungal mycelium cultures, especially in cases of late colonizer ECM fungi like *T. vaccinum*.

In sandwich culture experiments the ECM tree partner influenced the GST activity of the ECM system clearly, in respect to limited abilities for tolerance of toxicants.

As well as variations of GST activities between the parts of the plants were described by Anderson and Gronwald (1991). They found 25% higher GST activity in stems and leaves, whereas Maricela and Bernardino (2013) found opposite results with higher activities in roots and rhizosphere. It is conceivable that fungal symbiosis or contact may induce a more even distribution of GST within the plant. Or perhaps without the ECM fungal symbiosis partner, the GST activity would normally be higher in the plant parts, as Anderson and Gronwald (1991) described. Probably in presence of the ECM symbiosis, low GST amounts in the plant were needed to be required. Therefore less amounts are produced, due to the fungal interception of cell toxic substances by the help of the extrametrical mycelium and hyphal mantle. Hatton *et al*. (1996) found increase of GST activities in species over time. So under the adaptation process of organisms in their particular environment variations in GST activities can occur too. Furthermore the GST distribution also differs in young and older trees and the GST activities in plants differ between species over time. Therefore a difference in GST distribution between the plant structures in the soil and chlorophyll forming tree structures are conceivable.

T. vaccinum seemed therefore well adapted to the experimental used metal and radionuclide salt concentrations and with growth conditions on MMNb ½ with less lack of ECM partners. Very low GST activities and therewith decreased protection function regarding

the tree partner were consequences. The applied fungal strain *T. vaccinum* GK6514 was used since longer times under lab conditions and it is may to assume, that the ECM fungal ability to function as metal or radionuclide acceptor or catcher could be more and more lost or should be questioning. But still this fungus as late stage colonizer settles under natural conditions not either heaps nor HM or radionuclide loaded substrate and would therefore need no abilities for protection or interception in broader ranges.

Another point is the advantage of ecological adaptation of both early colonizer fungi in comparison to late colonizer *T. vaccinum* and its lack of accumulation or tolerance strategies for metal or radionuclide elements, focusing the GST activity. Possibly the GST activity is more costly for the fungal metabolism of late colonizer ECM or other mechanisms were used to protect the cells.

GST activity in young mycorrhizal tress confirms to data from the mesocosm experiments, performed with the two potential ECM tree partners *P. abies* and *P. sylvestris*. *P. sylvestris* showed not only low mortality rates at the used heap material, also measurements of root length showed clear differences. Longer primary roots of *P. sylvestris* showed 7 cm difference compared to those of *P. abies*. Secondary root length differed among 8-9 cm between the two trees. The other point is the plants dependency on ECM formation with the fungus. Root length data indicated as well as more branches occurred on mycorrhized roots of *P. abies* with 29% and lower effects occurred on *P. sylvestris* with 13% increase of root branches. These facts point out too, that *P. abies* tends to be more depending on the ECM formation, as symbiont of the late colonizer fungus *T. vaccinum*, compared to *P. sylvestris*, as symbiont of the early colonizer fungi *P. tinctorius* and *P. involutus*. Which could also explain high GST activities in C+S in the symbiosis between *P. abies* and *P. involutus* as possibly incompatible ECM symbiosis.

Dalton *et al*. (2009) described variations of GST forms in different plant structures. Possibly the measured conjugation reaction of CDNB limits the range of GST forms which can be detected. Many GST forms do not react to CDNB, so preferably a broader range of toxic substrates should be used. In different experimental set-ups a range of substrates including CDNB, ethacrynic acid and trans-4-phenyl-3-buten-2-one are used for testing microsomal GSTs (Marrs 1996, Datta and Samata 1992). There are potentially variations in GST activity between parts of the seedlings within the ECM fungal species too, especially under consideration of HM and radionuclide treatments. Experimental data show differences in GST activity despite HM and radionuclide treatments of early, *P. tinctorius* and *P. involutus*, and late colonizer fungi, *T. vaccinum*.

Mathematically a significant difference between the early and late colonizer fungi was found, but not between the two early colonizer species. Early colonizer fungi showed significant increased GST activity values compared to the late colonizer fungus. Maybe it is advantageous for early colonizer fungi to have higher potential for detoxification mechanisms while establishing the ECM symbiosis during the colonization. After Bellion *et al.* (2006) greater toxicity responses were required for HM contamination, like for cell protection. So with larger abilities to deal with toxins, early colonizer fungi can move into new environments which were not previously colonized. Bioavailable toxic compounds are easy accessible from initial organic layers. They are more often available in primary succession conditions, while in secondary succession an organic layer material is already established. Whereas high amounts of GST in late colonizer ECM fungi would be less advantageous, due to their lifestyle with later colonization and therewith secondary succession of areas. Therefore by the time, late colonizer species migrate areas which are already lowered in contaminants, conceivably removed by most of the early colonizer fungal species. The production of same amounts of GST enzymes from early and late colonizer fungi, could be unnecessary cellular expensive. Evolutionarily the amount of GST`s in cells of late colonizers would have been reduced over time in comparison to early colonizer fungi.

Occuring lacks of significance in the three sample types: fungal mycelium cultures, germination cultures and sandwich-cultures may due to the range of GST activities. The amelioration of HM and radionuclides by fungi is very depending on species, strain and type of HM (Godold *et al*. 1997). A way to improve the GST activity testing technique can be to analyze the GST enzymes in the ECM fungi, using molecular and genetic techniques. GST enzyme isolation as method to increase concentrations would be very beneficial, like GST purification methods described by Gronwald and Plaisance (1998). Homogenates of the sample were prepared, using extraction buffer to concentrate the material, application onto a gel-filtration column with subsequent elution. Alternatively a modified GST tag procedure could be used in future experiments, by using instead GST enzymes, proteins (Monti *et al*. 2005). Probable GST sequences are found in the genoms of the used ECM fungi *T*. *vaccinum*, *P*. *involutus* and *P*. *tinctorius* (Fig. 40). The sequences belong to the GST-C-family.

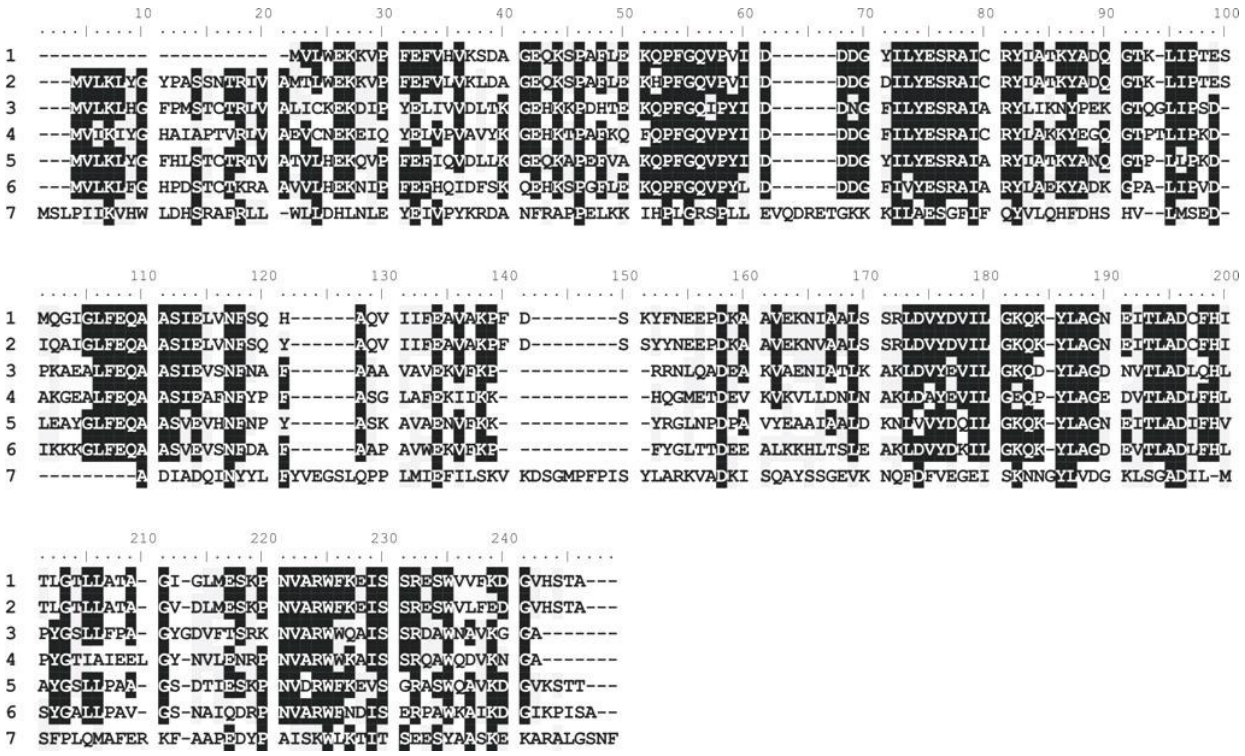

Fig. 40: **Alignment of GST protein sequences** of **1** - *Tricholoma vaccinum* g9224.t1, **2** - *Tricholoma vaccinum* g9225.t1, **3** - *Paxillus involutus* AAT91250.1, **4** - *Pisolithus tinctorius* KIN95264.1, **5** - *Laccaria bicolor* XP001888930.1, **6** - *Coprinopsis cinerea* XP001838582.1, **7** - *Saccharomyces cerevisiae* P40582.1 GTT1.

Heavy metal and radionuclide toxicity is a major constraint affecting root growth in ecosystems (Jenschke and Godbold 2000). Using the knowledge of GST activities in managed ecosystems such as in agriculture, may lead to greater understanding of fungal toxicity responses and therefore the possibility of increasing fungal and plants toxicity threshold especially in the ECM symbiosis. Relating to that, significant difference of GST activities between early and late colonizer fungi should be translated into agriculture and economical benefits for the amelioration of HM and radionuclides.

4.7. Guttation – the release of compounds and its physiological process

The generation of GD is a long known phenomenon in plants (Goatley and Lewis 1966). Therewith meant is overridingly the release or excretion of substances and by-products from plants and fungi. While for plants the maintenance of nutrient transport to avoid water saturation and interfered nutrient flow with a balanced tugor flow is well described (Weiler and Nover 2008). Fungal mycelial or fruiting-body exudation of GD and therewith its physiological function is not understood completely.

In GD from basidiomycetes, *L. lepideus*, products of the primary metabolism were found (Sprecher 1959). Mainly the sugars glucose and fructose as well as amino acids glutamine, valine, alanine and aspartic acid are verified in GD. But products of the fungal secondary metabolism were found too. Hutwimmer *et al*. (2010) described the occurence of destruxines from GD of the ascomycete *M. anisopliae* with known insecticide, phytotoxic and generally cytotoxic effects to eukaryotic cells. As well penicillin (Raper and Thom 1968) and ochratoxine (Gareis and Gareis 2007) was found in GD of different *Penicillium* species. McPhee and Colotelo (1977) and Colotelo (1978) describe GD as reservoir for primary and secondary metabolic products and as well as enzymes. Assumptions of Jennings (1991) determine GD as water reservoir to warrant the constant growth of areal mycelium. Georgiou *et al*. (2006) described in investigations of the basidiomycete *Suillus bovinus* a resumption or reabsorption of glucose into GD and therewith transport inside the pheripheral mycelium. And furthermore Georgiou *et al*. (2006) described connections between cell death and loss of membrane integrity.

Like in my investigations proven, the formation of GD could be a mechanism for selective excretion of cytoplasmic material (Sun *et al*. 1999, Georgiou *et al*. 2006). In the experiments used ECM fungi *Paxillus involutus*, *Pisolithus tinctorius*, *Tricholoma vaccinum*, *Tricholoma terreum* as well the saprophyte *Schizophyllum commune* showed different amounts of GD under several environmental conditions.

While *S. commune* was already established by Kniep, in 1920 and 1922, within its crossing experiments as model for genetic investigations, it is furthermore distinguished as model system for mushroom development through its short generation time (Jong *et al*. 2010) and fast production of GD, too. Within cultures of ECM and saprophyte fungi hints are found, that the process of guttation decreases under influence of stressors like permanent artificial light of 24 h or the influence of HM/Ra as well as fungicides like artificial strobilurin. The used *S. commune* double mutant Δ*ku8070* was described as being sensitive to UV irradiation with reduced growth and increased branching (Madhavan 2014). Increased formation of GD under biotic stress could have been a selective excretion, like Sun *et al*. (1999) recommends. GD of *S. commune* wt occur more often on primordia. This formation of primordi comes along with an increase of glucanases (Deacon 2006). This could be the hint for its cytoplasmic origin. Georgiou *et al*. (2006) assume, that exudates occur by losing membrane integrity and therewith occurrence while coincidence of cells. Sun *et al*. (1999) recommend this process as selective excretion of by-products.

The BSc project of Sehrt (2013) questions the role of high production of GD as answer

and sign for stress reaction a decreased production of GD in fungal cultures. It can even be possible, that the appearance of guttation is connected to the process of cell death and membrane degradation (Georgiou *et al*. 2006). Within dying cells a loss of the integrity of the membrane occurs and cytosol and cytosolic compounds can escape. Additionally the increase of glucanases, cell wall degrading fungal enzymes, while the cell death procedure is known (Deacon 2006). But these both approaches enhance the indication of guttation as a passive process and no active stress answer.

The positive test for D-ribose and L-glutamine, more clear for the *S. commune* mutant strain and less for the wt, can give more insides in functions of GST activity and exudation of GD. D-ribose is a pentose with five C-atoms. In nature it ocurrs with central functions in RNA and DNA, as energy carrier in: adenosintriphosphate (ATP), adenosindiphosphate (ADP) and adenosinmonophosphate (AMP) and secondary messenger substance. The proteinogenic free amino acid L-glutamine is a universal vector of amino groups especially for biosynthesis of purin and histidin. L-glutamine is furthermore part of the glutathione tripeptide GSH which consists of L-glutamine acid, L-cystein and glycin. This enzyme was found and proven in exuded droplets and can the same time being part of the GST activated scavenging process of cell toxic substances and nucleophilic bounds to the thiol group of the GSH. So these connection can be interpreted as scavenging process of toxic elements in the cytosol to avoid damages and promote exudation of elements *via* exudation of GD. The forms of elemental exudates, if they were bound or present as radicals, needs to be proven furthermore. L-glutamine is present in the cytosol of fungal cells. Both, D-ribose and L-glutamine, were detected in GD.

Strobilurine, naturally produced by *Strobilurus tenacellus* as strobilurin A and synthetic applied as agricultural fungicide (Moore *et al*. 2011), is naturally unstable in presence of day light and caused high amounts of GD with clear values of sugar and amino acid therein.

Young extramatrical mycelium extends permanently in fresh nutrient zones (Deacon 2006). But GD occur mainly in older parts of fungal mycelium or fruiting bodies. Additionally was found, that early colonizer ECM, like *P. tinctorius* together with *P. involutus* and *T. vaccinum*, produced 1/3 increased amounts of GD together with other ECM fungi compared to separate cultures. This could be one more hint for the early protection of germs and young seedlings by ECM early colonizer fungi. Especially under consideration of increased production and element amounts of early colonizer ECM fungi GD compared to late colonizer.

Pb blocks free thiol groups, especially enzymes of the porphyrin synthesis. Targeted guttation experiments with 0.5 and 1 mM $PbCl_2$ media showed, that *P. involutus* exuded very high amounts of Pb within GD Also GD showed increased dark brown colored pigment production on air mycelium on 1 mM $PbCl_2$ media after 6 months.

The ECM fungi mainly produced in the same time less amounts, around a quarter lesser, of GD than *S. commune*. Even if the droplets showed less pigmentation, but generally, depending on the fungal species, older fungal cultures produce more pigments in the mycelium, medium and in the droplets too (Aloj Totaro *et al*. 1986, Abo Ellil 1999).

By facing the question about the structural origin of GD, a closer look to the hyphal ultrastructure is necessary. The cell plasma membrane is capable for endocytosis (uptake) and exocytosis (secretion). Especially the endocytosis processes, with active membrane assistance, provides the option for the uptake of essential metabolites, some regulators and growth factors or previously exported molecules for recycling. Coated pits on the outside of the membrane, to transport endocytic vesicles to the Golgi apparatus, endoplasmic reticulum (ER) and fuse with Golgi and ER endosomes are taking place in specialized regions (Moore *et al*. 2011). Whereas the exocytosis requires growth of fungal cell wall and plasma membrane. So this process provides vesicles for cell wall growth and extension as well as induces extracellular enzyme secretion (Bartnicki-Garcia *et al*. 2015). The exocytosis is localized at the hyphal apex and the endocytosis takes place subapical collar (see Fig. 6).

Physiologically onto the Spitzenkörper hyphae have a polarized growth with some tip-directed transport of secretory vesicles (Fisher-Parton *et al*. 2000). In this temporarily accumulation zone of the hyphae (Fig. 41), the exudation of cytoplasmic material and within by-products are excreted by the exocytosis process (Riquelme *et al*. 2014). The secretory process of exocytosis, with a fusion of secretory vesicles which entails to the plasma membrane, serves as key for cell growth and morphogenesis (Shandala *et al*. 2012). In the exocytosis process the vesicle membrane fuses with the plasma membrane by a fusion pore that dilates until the secretory vesicle collapses into the plasma membrane and the contents of the vesicles are released into the extracellular space (Rizzoli and Jahn 2007) and by this way into the surrounding environment. Alternately GD were formed and exuded on hyphal septa (Fig. 6) and septa expected zones (Fig. 41). In this zone, while growing processes of hyphal branch formations including new septa formation (Riquelme and Sanchez-Leon 2014), an exudation of GD was shown.

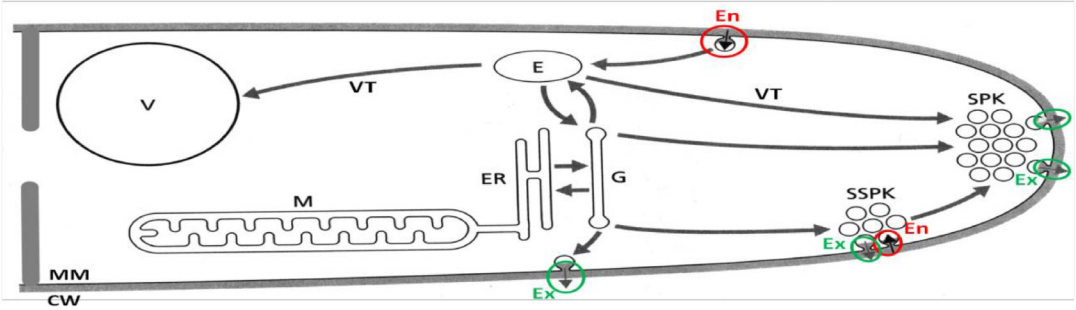

Fig. 41: **Model of vesicle trafficking with endocytosis and exocytosis in growing hyphal tip**. **CW** – cell wall, **E** – Endosome, **ER** – endoplasmatic reticulum, **En** – endocytosis, **Ex** – exocytosis, **G** – Golgi cistern, **MM** – membrane, **M** – mitochondrion, **SPK** – Spitzenkörper, **SSPK** – satellite Spitzenkörper, **V** – vacuole, **VT** – vesicle trafficking. Modified after Fischer-Parton *et al*. 2000.

Concerning the physiological process of exudation the vesicle trafficking at hyphal tip regions can provide some mechanistic understanding. So it permits on one hand the guttation process, as exocytosis, and implies on the other hand the absorption of hyphal closely substances, by endocytosis, which serves for up taking nutrients *via* ion channels. At the growing tip of the fungal mycelium an electrical field becomes generated around. The exterior of the hypha is more electronegative at the apex. There the entering inside the tip is possible. This electrical field seems to be intimately involved in nutrient uptake. While the nutrient uptake is an energy-dependent process, ion pumps and symport proteins act in closely. The nutrient uptake symporters are active at hyphal tips. In this case sugars, as carbohydrates and essential C-sources and nutrients for fungal growth, are provided in fungi. Especially important for the extramatrical mycelium and fruiting body formation. (Barker *et al*. 1998). Fungi rely on the considerable extent with exudation of by-products across plasma membranes and cell walls, which than act as molecular sieves (Sun *et al*. 1999). Hyphae and rhizomorphes draw water with minerals and its bondings from the soil, the organic layers also distances away, up into the hyphae, hyphal aggregates and fruiting bodies. There the elements like HM or Ra can be accumulated.

Furthermore the option to exude dissolved organic compounds, like the nutrients D-ribose and L-glutamine, mineral ions and by-products like toxic compounds, occur in GD (Turnau *et al*. 1994).

The essential element Ca is an intracellular messenger substance, signaling system and involved in tip growth (Jackson and Heath 1993), as long as tips require extracellular Ca for continued tip growth. The plasmalemma includes at the very tip of the hyphae high concentrations of Ca-channels which activate while cell stretching. So Ca is disposed for the

ingress when membrane stretches (Deacon 2006). But Ca also act as second messenger in signaling in early steps of ectomycorrhizal infection (Levy and Bres 2004, Harrison 2005). Moreover Ca generally is stored in vacuoles, ER and mitochondria for further applications of hyphal tip growth and intracellular regulation of free Ca levels of calmodulin. This calcium-binding protein, is known to be very important for complexation of Ca to form hyphae and in consequence hyphal strands. (Deacon 2006). Ca, as opponent to Pb, was exuded in high amounts in about double values in GD of *P. involutus* and *P. tinctorius* within Pb-treatments. While in co-cultures with more than two fungi in one culture plate, the Ca exudation was decreased and approximately similar to the Ca excretion in the control.

The cross walls of basidiomycetes, the septa, divide the hyphae in regular intervals. Else septa have pores for cytoplasm and organelles exchange. So the fungal hyphae are interconnected compartments which offers the option for cell components to move forward and backward, especially elements too. The fungal chitin wall, with the matrix components mannoproteins and alpha 1-3-glucan, which surrounds hyphae, is thin at the apex (~ 50 nm) and thickens up to 125 nm behind the tip (Deacon 2006). At the apical tip of the hyphae, the Spitzenkörper is placed. At this Spitzenkörper an exudation of inner cell material can take place through vesicle trafficking and the exocytosis (Deacon 2006). The fungal wall is the important gate to the environment. So it protects against osmotic lysis and UV, acts as molecular sieve while regulating the passage of large molecules through the wall pore space or lysis enzymes of other organisms.

With regard to remediation aspects, the Pb content in medium, fungal mycelium and GD was investigated. The most Pb amount in GD was detected from the ECM fungi *P. tinctorius* and *P. involutus* compared to their mycelium and medium. In contrary high Pb amounts of *S. commune* cultures were found in the media, lower in mycelium and GD. This links to the consideration, that *S. commune* generally do not absorb HMs like Pb intracellularly.

Taking a look on a larger scale the cultures condensation water was investigated, next to the GD, in one culture of the fast guttation producing fungus *S. commune* (Fig. A1, A2 appendix). It was found, that even in condensation water of *S. commune* Pb was detected in minimal amounts. While Ca was present in much higher amounts. While Ca was increased under normal conditions and seemed to decrease in Pb treatments. Only the *S. commune* mutant strain showed a reverse behavior. All in all is to note, that Pb is delivered by condensation water, the same as Ca, but in case of *S. commune* mainly in traces. While condensation with prior accumulation of elements of *Amanita* species was described for

vanadium too (Rehder 2013). What therein could be explained, that *S. commune* is not taking up and for this reason does not excretes substantially amounts of Pb. For more clear informations the experiment about condensation of elements should become expanded to ECM cultures.

These facts can be interpreted as molecular sieve function of the guttation process but as fertilizing and dispersal function else. So the excreted cytosolic GD reaching the ground under natural conditions and the exuded substances can become released (Fig. 43). These released substances are free for further dispersal and bounding in the surrounding organisms. Especially organic compounds and nutrients are than available for new growing mycelia or other ecosystem establishing and obtaining members. Even indirectly by providing C-sources attract therewith potential mating partners.

Studies by Hutwimmer *et al*. (2010) have shown, that the content of GD of filamentous fungi interplay with the nutritional effects given by cultivation media. So it was proven, that GD under laboratory conditions were formed if the fungus grow on media containing two carbon sources. A HPLC result therewith revealed, that the content of droplets is similar in sugar and acid. (Hutwimmer *et al*. 2010). Also Georgiou *et al*. (2006) agreed with the fact that fungal exudates are possibly of cytoplasmic origin. Sun *et al*. (1999) described that exuded droplets result from selected excretion of cytoplasm to cope with waste products. This hints to the molecular sieve functioning.

This directly can be connected to my results of high exudation of elemental Pb and Ag. Whereby both elements are targeting the inhibition of thiol groups. Unestam and Sun (1995) found high amounts of nutrients were readily reabsorbed after exudation while by-products were not. This represents regulated interface exchange between peripheral hyphal tips and their immediate environment. Moreover Hutwimmer *et al*. (2010) announced that exuded fluid can influence the composition of bacterial communities associated with hyphal tips. These fungal strategies give the option to survive optimally.

One approach for the movement of substances like elements from the inner part of the cell to the external *via* water fluxes, in form of GD, are water channels like aquaporins (AQP). Marjanovic *et al*. (2005a, 2005b) found, that the formation of AQP tends to be enhanced which could preserve vitality under water stress conditions. In relation to root water flow properties, increased AQP function and increased root hydraulic conductivity in ectomycorrhizal plants are described. The AQP function of the fungal hyphae is also likely to be important for the uptake of water by the ectomycorrhizal plant (Lehto and Zwiazek 2011).

Previous studies of Unestam and Sun (1995) found, that water transport properties

depend on hydrophilic and hydrophobic characteristics of the external hypha. Hydrophilic ECM fungi, like different *Laccaria* and *Lactarius* species, transport water into the apoplast. While hydrophobic fungi, like *Paxillus involutus* and *Suillus* species, form mycelial cords for water transport into the symplast. In this case the hydrophilic part of the mycelium is limited to the hyphal tips, which contacts to soil water, and only in this hyphal region the uptake of water and solutes proceeds. Which could be the reason for the resulted low amounts of GD under influence of AQP inhibitor acetazolamide (AZA). The same occurs with *T. vaccinum* under influence of $PbCl_2$, AZA and Ag salt. Even if *T. vaccinum* produces generally very low amounts of GD, this reaction was strengthened. In case of *P. tinctorius*, very high volumes of GD were exuded under Ag influence and very low under AZA. It seemed also like *P. tinctorius* AQP were mostly affected by AZA and Ag. Both induced a faster transport like "cleaning" of the cell. As well it could be, that Ag acted very aggressive and induced AQP channel damages. Which needs to be proven further.

The mechanism of Ag inhibition is most likely due to their ability to interact with sulfhydryl groups of proteins (Montalvetti *et al*. 2004). Mechanistically inhibits Ag actively Na^+ and Cl^- uptake. Phosphates, carboxyl amino acids, sulphate groups, metals or other cations (Ca^{2+}) are additionally competitively bound by Ag^+. (Ratte 1998).

But the permeability of membranes for water and solutes needs a driving force of its movement, which is ensured by the osmotic-chemical potential of both membrane sites (Finkelstein 1987), potentially for both directions. The water-solutes flux across membranes by diffusion acts *via* two ways: over the hydrophobic bilayer and the proteinaceous pores of molecules. This indicates lower activation energy for the transport, but varies depending on surrounding temperature (Nehls and Dietz 2014). Even if the nine functionally characterized fungal members showed minor permeability for solutes and more for water, although fungal AQP can be permeable for gases and solutes (Navarro-Rodenas *et al*. 2011). But from genome analysis of 480 fungi 41% had AQP, only for water facilitation, and 49% aquaglyceroporines, for transmitting as well as solutes. The last members form therewith a phylogenetic distinct group. (Nehls and Dietz 2014). Cheng *et al*. (2013) detected a aquaglyceroporin-encoding gene in *Coprinopsis cinerea* in primordial stages of fruiting bodies, but no AQP-encoding gene in the genome.

In own investigations different AQP1, AQP4 and aquaglycerporin sequences were identified based on data of *Gloeophyllum trabeum* (XP007867203.1; Floudas *et al*. 2012), data of *Caenorhabditis elegans* Aqp-1 – Aqp-8 (Huang *et al*. 2007) and with *Plasmodium*

falciparum aquaglyceroporin PfAQP (Newby *et al*. 2008). Hereupon three sequences were detected in the genome of *Tricholoma vaccinum* (Fig. 42).

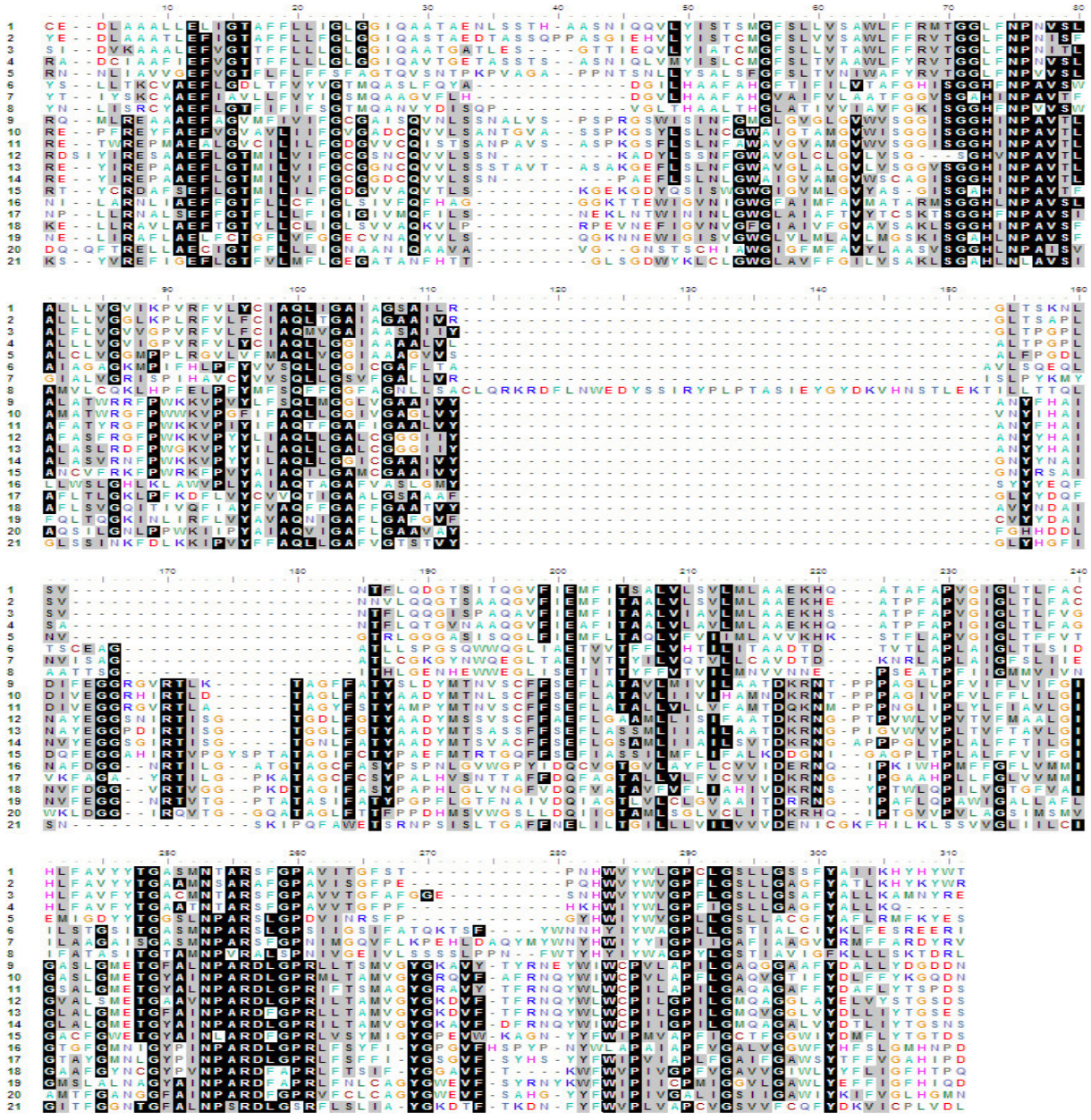

Fig. 42: **Partial alignment of AQP and aquaglyceroporin proteins** of **1** - *T. vaccinum* g9123.t1 (position 11-240), **2** - *L.bicolor* AFJ15555.1 (position 13-243), **3** - *P. involutus* KIJ14435.1 (position 17-246), **4** - *G. trabeum* XP007867203.1 (position 19-242), **5** - *A. fumigatus* XP746526.2 (position 19-242), **6** - *C. elegans* NP505512.3 Aqp-4 (position 41-271), **7** - *C. elegans* C32C4.2 Aqp-6 (position 8-242), **8** - *C. elegans* CAA94903.2 Aqp-5 (position 40-314), **9** - *T. vaccinum* g2094.t1 (position 51-308), **10** - *L. bicolor* AFJ15558.1 (position 41-298), **11** - *T. vaccinum* g73.t1 (position 53-310), **12** - *P. tinctorius* KIN96312.1 (position 46-296), **13** - *P. tinctorius* KIO03273.1 (position 108-366), **14** - *P. involutus* KIJ17815.1 (position 49-300), **15** - *A. fumigatus* KEY82230.1 (position 54-308), **16** - *C. elegans* CCD66277.1 Aqp-1 (position 20-271), **17** - *C. elegans* NP508515.2 Aqp-7 (position 19-269), **18** - *C. elegans* CAA84642.1 Aqp-2 (position 14-261), **19** - *C. elegans* Y69E1A.7a Aqp-3 (position 117-369), **20** - *C. elegans* NP001024758.1 Aqp-8 (position 16-266), **21** - *P. falciparum* 3D7 XP001348009.1 aquaglyceroporin PfAQP (position 7-248).

Two of them, g9123.t1 and g2094.t1, are similar to AQP1 and AQP4, one, g73.t1, resembles the aquaglyceroporin.

The first eight sequences in the alignment of Fig. 42 tend to fit with AQP1 and *C. elegans* Aqp-4, 5 and 6. The sequences nine to 21 show stronger similarity to *C. elegans* Aqp-1, 2, 3, 7, 8 and *P. falciparum* aquaglyceroporin PfAQP (Fig. 42). All sequences enclose the motifs of MIP superfamily, the amphiphatic channel and the Asn-Pro-Ala signature.

So maybe the theoretically hypothesis of strictly separation of AQP for water transport and aquaglyceroporins for solute transport only, should become rethought. It is conceivable, that AQP in fungi, especially in ECM fungi, can take over their respective functions. The better effectiveness of water use of ECM fungi is proven indirectly by significant improve of water efficiency of ECM plant. This indicates a decreased water demand while the symbiosis. (Mushin and Zwiazek 2002 a, b). Why should this not function in both directions? For the ECM fungus *Laccaria bicolor* Dietz *et al*. (2011) found aquaglycerporins in the fungal plasma membrane to be permeable for ammonia transfer. The presence of AQP could increase water flux *via* the plasma membrane and promote the turgor development (Nehls and Dietz 2014).

Whereby even tendencies were found that elements which are part of GD can become physically released by evaporation or physiologically by cell respiration. Transferred to fungal and hyphal environments, the emitted traces of elements can entering the soil parts water phase, air phase or solid phases. Secreted vesicles can fuse with the plasma membrane and empty contents into their extracellular space (He and Guo 2009, Jahn and Südhof 1999). This is substantial important for delivering cell wall-synthesizing enzymes, membrane proteins and membrane lipids to the apical plasma membrane, for example during tip growth (Wessels 1993). After Hayakawa *et al*. (2011) membrane associated proteins, the SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) facillitate therefore docking and fusion of vesicles with target membranes. Wösten *et al*. (1991) and Schultzhaus and Shaw (2015) described already, that enzymes are secreted from hyphal tips, but not only at the periphery of the mycelium. Hyphal tips and Spitzenkörper regions with septa are locations of active hyphal growth and assembly points for exudates and compounds. So high amounts of Ca in GD can be due to the fact of growth while Ca functions as co-factor for different proteins (personal communication R. Mourino-Perez, Dept Microbiology CICESE, Ensenada, Mexico). Pb could therewith additional be exuded. As well as a central question after the investigation of C-sources, amino acids, Ca and Pb in GD occurs: What could the guttation mean for the fungus and which relevance comes along?

Under the aspect of releasing next to expected by-products like Pb and Ag, precious organic and essential compounds like D-ribose, L-glutamin and Ca the idea about fertilizing suggests itself (Fig. 43). Maybe GD conduces the development of fungal mycelium, of the own fruiting body, to explore a larger field and the directly surrounding area, in general for self-preservation. But the same time it can serve for contacting and attract mating partners. Under the perspective of releasing secondary metabolites ochratoxins and other mycotoxins (Gareis and Gareis 2007) by guttation, it is may possible to understand the guttation as a form of communication and so also protection of the host plant against herbivores or other the plant and therewith ECM system possibly wounding grub enemy. This could consequently receives and ensures the nutrient source of the fungus. But on the other hand fungal mycelia and fruiting bodies, especially ECM, are able to take up and accumulate high amounts of heavy metals and radionuclides. To prevent self detoxification, the hyphae maybe help oneself by exude surpluses of elements out of the fungal mycelium up to specific thresholds. Which conduce not only little fungal parts, but the whole hyphal system, due to the aspect of pores in septated hyphae. So the physiological construction of the fungal hyphae allows to accumulate high amounts of non-essential substances and elements. The same time the fungal hyphae are able to release surpluses of elements from the whole hyphal strand. The fact that Pb can replace Ca physiologically can be important if an overload of HM occurs inside the fungal cell. Through this vitality and metabolism stabilizing processes, like cell stretching, hyphal tip growth and the formation of hyphal strands, can be interrupted through low Ca level.

Furthermore Ca and Pb are both stored in vacuoles, Ca as reservoir and Pb for detoxification. To come closer to some hint what the exuded Pb could maybe mean for remediation, phyto- or mycoremediation, it could be concluded that exudation of elements, substances and by-products, can serve a dispersal option. In intact balanced ecosystem structures released substances are intercepted by other contact partners of the ecosystem and can further have new functions in the ecosystem consortium. Accordingly all three fungi together: the uptake and exudation of Pb from one ECM fungus increases up to nearly one third more by co-working with two more ECM fungi.

But the ECM fungus seems to use processes too, which act against the lone some single sequestration of HM and Ra. The formation of GD and implied exudation of substances could be more conductive for cell detoxification and therewith promoting elemental distribution. With the exudation of toxicants, by-products like HM and Ra or substances, *via* GD a detoxification of the cytosol could be grant for at least self-preservation of the organism at the respective substrate.

4.8. Conclusions - sequestration and path of metals through ectomycorrhiza

The hypothesis ECM association influences the sequestration of HM and radionuclides could be confirmed within this work. While the ECM diversity can the same time influence the mobile metal concentrations. In mesocosm experiments with soil from loaded sides obviously changes in element availability, not least caused by ECM interactions, and root length diversity was seen. Cause mycorrhizal fungi are substantially involved in mineral transformations and distributions of especially inorganic nutrients, they can dissolve minerals and mobilize elements very efficiently (Burford *et al*. 2003; Gadd, 2004, 2007). So released elements, metal complexes or metal ions can interact with biomass. Hyphae can drive these processes by providing organic acids and anions. The morphotyping of ECM correlates therewith to the sequestration of elements.

Prescott and Grayston (2013) described clearly that mycorrhizal fungi in association with tree roots can influence the soil and soil microbial community. Especially ECM mycelia can yield about 80% of the fungal community and contribute up to 30% of the total microbial biomass (Högberg and Högberg 2002, Wallander 2006). The applicability for remediation of *Pisolithus tinctorius*, *Paxillus involutus* and *Tricholoma vaccinum* was proven in mesocosm experiments. Whereat the different fungal diversity depends on tree species associates (Buee *et al*. 2011) and can modify the ECM community by inoculation. Additionally the ECM diversity can change the mobile metal concentration in the soil. This was proven by the removal of the elements Cs, Pb, U, Sr, Ni and Cd from the bioavailable and soluble soil fraction of determined mesocosm experiments.

This relates directly to the point of exudation of nutrients like sugars and amino acids as fertilizing and increase factor for tree populations. Within this, the root surrounding soil area needs to become focused. This rhizosphere is very rich in and accommodates a diverse range of organisms and root associatives. This area is too, very rich of diverse exudates and growth regulators (Prescott and Grayston 2013). Heinonsalo *et al*. (2004) and Jones *et al*. (2004) described both the excretion of carbohydrates and organic compounds which are used by microorganisms in the surrounding of the extramatrical hyphae and Nazir *et al*. (2010) emphazised this region as “hotspot” for bacteria. As well ECM root tips are described as supporters of bacteria and soil fungi populations in connection with stimulation and attracting of new fungal associates (Frey-Klett *et al*. 2007, Finlay 2008, Labbe *et al*. 2014). Rhizomorphes are active sites for exudation and re-adsorption of different compounds and substances (Sun *et al*. 1999). So this can be a field for accumulation of elements on one hand

and exudation of guttation compounds and by-products on the other. But also the permeability of the fungal membrane needs to be considered.

Yin *et al*. (2014) hypothesized, that trees with ECM associations exude more C than tree species in other associations. As well for nutrient poor conditions Lindahl *et al*. (2005) described the increase of nutrient availability by ECM fungi through excretion of enzymes with subsequent recycling (Read and Perez-Moreno 2003) too. Moreover this can result in enhancement of nutrient cycling in ECM rhizosphere. And also cytosolic detoxification systems like the GST activity are involved inside the cell in ECM establishment and persistence. The activity of enzymes, like the cytosolic GST, can be affected by HM and Ra influence. Due to the organisms affinity to functional groups, like the SH-groups. Thereby the appearance of GD seems to play in this connection a substantial role for the translocation of nutrients, enzymes, secondary metabolites and elements. By this way an exclusion of other organisms, like saprotrophes, from energy–depleted substrates, can proceed. Especially to monopolise nutrients for themselves and their hosts. (Lindahl *et al*. 2007). Influences of stressors on growth of fungal mycelium can affect the occurrence of GD too.

The activity of enzymes can be derogated or damage membranes. Which function occurs from the fungal exocytosis? Firstly the exocytosis is directly responsible for growth of cell wall and plasma membrane. The process of exocytosis at the hyphal apex is described as highly localized. (Bartnicki-Garcia *et al*. 2015). But it creates an excess of plasma membrane and thus the need for removal by endocytosis while the growing process too. Moreover requires the exocytosis vesicles for cell wall extension and extracellular enzyme secretion. After own observations GD occurred along hyphal strands like a pearl chain (*P. involutus*, Fig. 22B) and directly at septa (*S. commune* wt, Fig. 22C), additionally to the tips (*P. tinctorius*, Fig. 22A). Can the process of guttation be seen as exocytosis? Is exocytosis an active process? Out of view of the ECM: If guttation is an active process, could it influence the environment by exudates and fertilizes to attract other interaction partners, for nutrient cycling, potential fungal plant partners, biomass production within sucession of ecosystems.

A hypothesis of the benefit of fungal guttation can be described: Water, minerals and elements can be drawn from the soil and far distances. The uptake follows directly with and a cytosolic movement too. Subsequently the exudation of GD can occur. And therewith connected the release of H_2O enriched with organic compounds, by-products, mineral ions and elements. By this pathway the environment is fertilized, to attract if applicable other organsims like bacteria, fungi or plants. The surrounding biomass can be increased for potentially further nutrient sources (Fig. 43). But the same time metal stress can be reduced by

the release of elements out of the cell, connected with dilution effects.

To fundamentally establish the hypothesis of environmental feeding and exuding of proportional amounts of elements like Pb: a fungal succession during litter decomposition or biomass production and accompanied stratification of microbial communities correspond to soil forming layers especially in forests (Grayston and Prescott 2005, Snajdr *et al*. 2011, Baldrian *et al*. 2012). A potential pathway of fungal guttation water from occurrence into its environment can possibly being understand as fertilizing path. By drawing soil water, minerals and metal elements from deeper soil regions and distances, hyphae can fertilize the surface and upper soil layer by the process of guttation. So the release of GD with its including compounds and by-products can result in the attraction of other organsims like bacteria, fungi or plants and thereby an increase of surrounding biomass comes along. Additionally element contents are bound and sequestered in the biotic surrounding by these transfer processes.

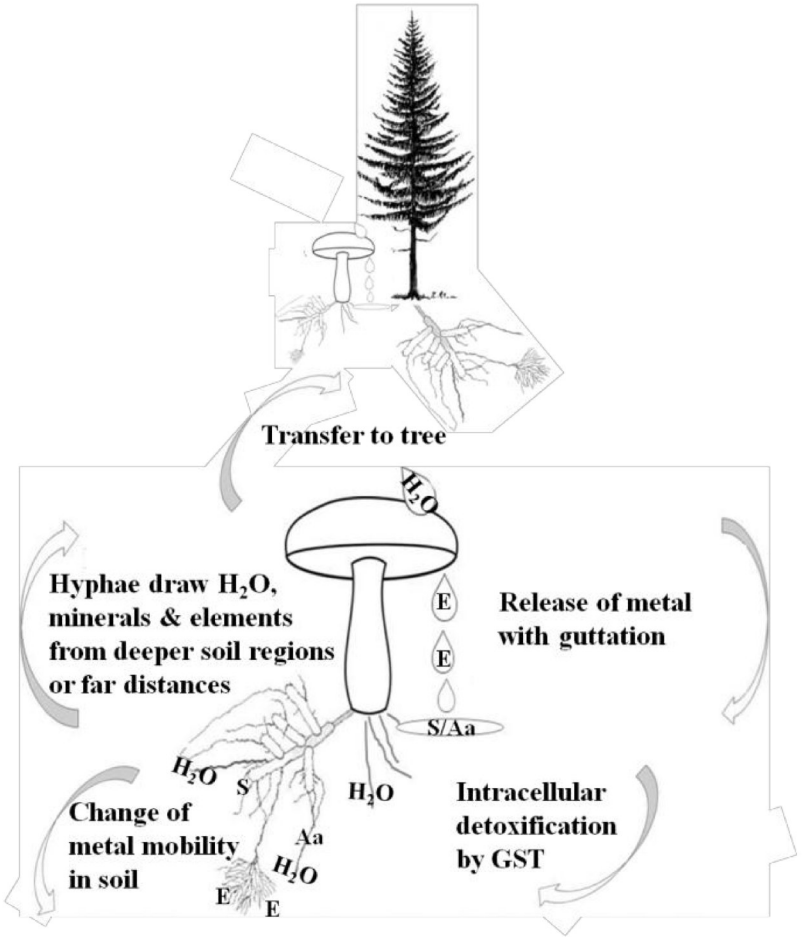

Fig. 43: **Pathway of fungal guttation water from occurrence into its environment**. **Aa** - amino acids, **E** - mineral elements, **H_2O** - water, **S** - sugar.

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6. Appendix

Tab. A1: **Calculation of elemental Pb amounts in 1 L MMNb ½ medium**, 1 petri dish (1 replicate) and 3 petri dishes (3 replicates).

Concentration	Pb amount in medium
1 mM $PbCl_2$	280 mg/per L
	7.78 mg/25 ml (1 replicate)
	23.340 mg/75 ml (3 replicates)

Tab. A2: **Data of Pb content from guttation droplets of 1 mM $PbCl_2$ cultures and control for each fungus in mg/g**. Legend: C – control without $PbCl_2$ addition, S – single mycelia culture, T – three mycelium cultures together, Co – three different ECM fungi together in one co-culture.

	P. tinctorius			***P. involutus***				***S. commune* 12-43 x 4-39**		
	C	**S**	**T**	**C**	**S**	**T**	**T in Co**	**C**	**S**	**T**
Med	0.00028	1.4	4.2	0.0002	1.4	4.3	3.7	0.00034	3	9
Myc	0.00074	0.048	0.144	0.00119	0.37	1.1	0.9	0.00018	0.123	0.37
GD	**0.1**	**3.9**	**11.8**	**0.2**	**6**	**18**	**25.8**	**0.02**	**0.17**	**0.5**

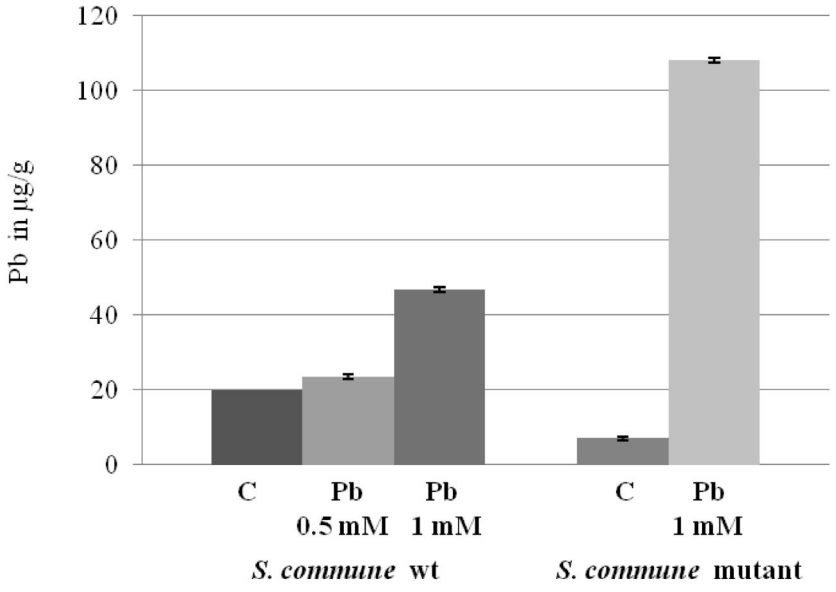

Fig. A1: **Pb content of condensation water of *S. commune* wt and mutant strain cultures under 0.5 and 1 mM $PbCl_2$ treatment**. Legend: C- control without $PbCl_2$, Pb 0.5 mM – 0.5 mM $PbCl_2$ in MMNb ½ medium, Pb 1 mM – 1 mM $PbCl_2$ in MMNb ½ medium.

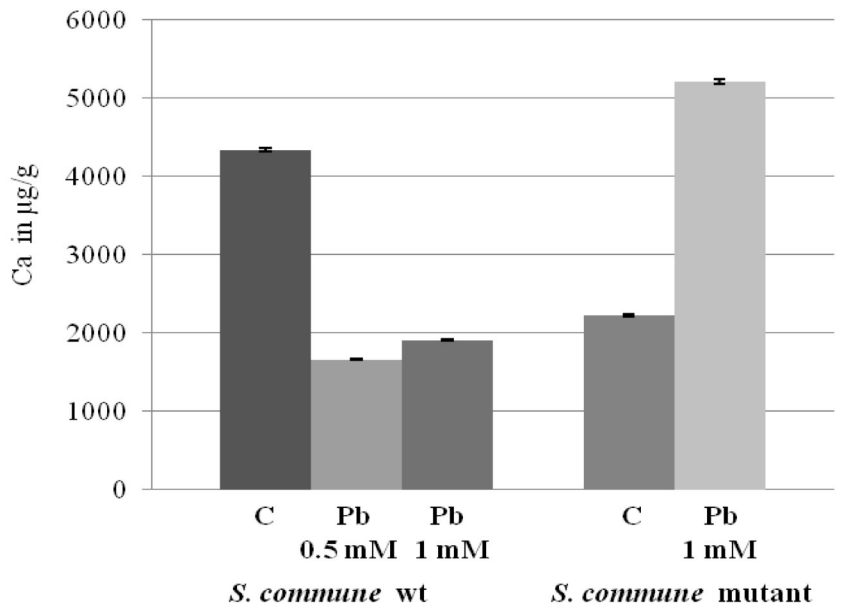

Fig. A2: **Ca content of condensation water of *S. commune* wt and mutant strain cultures under 0.5 and 1 mM $PbCl_2$ treatment**. Legend: C- control without $PbCl_2$, Pb 0.5 mM – 0.5 mM $PbCl_2$ in MMNb ½ medium, Pb 1 mM – 1 mM $PbCl_2$ in MMNb ½ medium.

Tab. A3: **Data of Ag, Pb, Cu, Mn and Si content of guttation droplets in µg/g**. Legend: **C** – control (separate) culture on MMNb ½, **2/3 Co** – *P. involutus*, *P. tinctorius* and *T. vaccinum* together in co-culture, **Pb** – with 0.5/1 mM $PbCl_2$, **AZA** – with 0.01 mM acetazolamide and **Ag** – with 0.01 mM AgCl or $AgNO_3$.

		Ag	**Pb**	**Cu**	**Mn**	**Si**
		µg/g	µg/g	µg/g	µg/g	µg/g
P. tinctorius	**3 Co Pb + AZA**	0.003	0.217	0.165	1.59	2.95
	Pb + AZA	0.004	0.062	0.194	1.81	6.1
	3 Co Pb	0.004	0.002	0.203	0.365	17
	2 Co Pb	0.003	0.206	0.067	0.966	7
	3 Co C	0.003	0.008	0.092	2.32	9.11
	2 Co C	0.003	0.001	0.075	1.5	22
	C	0.003	0.001	0.074	2.2	7
P. involutus	**Pb + Ag**	0.028	1.253	0.09	1.1	12
	3 Co Pb + AZA	0.012	0.394	0.485	9.1	10
	2 Co Pb + AZA	0.02	1.76	0.155	1.272	16
	2 Co Pb	0.003	0.29	0.906	0.68	5.79
	Pb	0.003	0.201	0.166	0.4	12.7
	C	0.003	0.001	0.26	0.518	8.7

Tab. A4: **Data of Mg, Ca, S, Na, K and P content of guttation droplets in µg/g**. Legend: **C** – control (separate) culture on MMNb ½, **2/3 Co** – *P. involutus*, *P. tinctorius* and *T. vaccinum* together in co-culture, **Pb** – with 0.5/1 mM $PbCl_2$, **AZA** – with 0.01 mM acetazolamide and **Ag** – with 0.01 mM AgCl or $AgNO_3$.

		Mg	**Ca**	**S**	**Na**	**K**	**P**
		µg/g	µg/g	µg/g	µg/g	µg/g	µg/g
P. tinctorius	**3 Co Pb + AZA**	11.7	18.5	4922	57	86.08	84.9
	3 Co Pb+AZA	13	21.9	4632	58.3	91	97
	Pb+AZA	18.2	23	9027.7	104.15	113	153
	3 Co Pb	39.8	16.4	16	109.5	260	82
	2 Co Pb	9.82	13	9	34.1	100	79
	3 Co C	12.4	21.6	9	43	71	109
	2 Co C	16.13	25	20	65.7	77	141
	C	29	31.5	54	121.4	179	225
P. involutus	**3 Co Pb + AZA**	91.2	174.4	4680	121	157	97
	2 Co Pb + AZA	13.7	20.4	8175	118	123	100
	Pb+AZA	4.31	4.8	68	42.4	85	49.3
	Pb + Ag	9.876	15.3	147.6	64	124	88
	2 Co Pb	8.76	7.5	27.51	31.4	85	72.7
	C	8.974	8.1	36.5	33.9	105	83

Tab. A5: **Data of non-essential and essential elements of heap material in 3 cm depths**. Legend: O I/II/total – original soil fraction I, fraction II and total extraction of fraction I-VII after Zeien and Brümmer. I – mobile bioavailable, unspecific absorbed fraction; II – easy deliverable fraction. C I/II – control soil fraction I and II. M I/II – with mycorrhiza fraction I and II. P I/II – plants without mycorrhiza inoculation fraction I/II.

µg/g	**O I**	**O II**	**O total**	**C I**	**C II**	**M I**	**M II**	**P I**	**P II**
Al	105.4	6.15	81293	187	< 6	119	6	162	6
				217	< 6	139	6	164	6
				173.2	< 6	120	6	162	6
						103.75	6	132	6
						99	6	115	6
						77	6	125	6
						93	6		
						103	6		
						116.7	6		
						95.9	6		
						99	6		
						109	6		
Cs	0.0906	0.015	3.33	1.064	0.145	0.819	0.097	0.9	0.115
				1.02	0.142	1.01	0.139	1	0.123
				0.953	0.126	0.769	0.094	1.08	0.137
						0.535	0.056	0.729	0.08589
						0.631	0.07	0.585	0.068
						0.417	0.049	0.918	0.109
						0.6642	0.079		
						0.5675	0.064		
						0.8527	0.109		
						0.545	0.066		
						0.6466	0.0756		
						0.98	0.13		
Pb	0.08182	0.04	29.37	0.069	0.022	0.111	0.0258	0.14	0.028
				0.057	< 0.012	0.0424	0.0151	0.2099	0.0425
				0.06	< 0.012	0.0552	0.0147	0.1055	0.021
						0242	0.0516	0.032	0.012

Pb						0.0921	0.025	0.0664	0.012
						0.0465	0.032	0.042	0.012
						0.0876	0.028		
						0.1167	0.03561		
						0.066	0.017		
						0.06756	0.0216		
						0.084	0.0217		
						0.09	0.0232		
Sr	0.719	0.105	131.4	0.86	0.0943	1.214	0.116	0.747	0.095
				0.831	0.1006	1.41	0.141	0.8499	0.097
				0.757	0.088	1.125	0.115	0.997	0.112
						0.968	0.096	1.045	0.105
						0.8565	0.094	1.345	0.127
						1.23	0.139	1.34087	0.21
						1.164	0.109		
						1.226	0.125		
						1.162	0.1177		
						1.262	0.133		
						1.341	0.161		
						1.712	0.187		
Cd	0.00519	0.004	0.37	0.01	< 0.012	0.01	0.012	0.0094	0012
				0.0075	< 0.012	0.0127	0.012	0.009	0.012
				00105	< 0.012	0.008	0.012	0.011	0.012
						0.0086	0.012	0.0096	0.012
						0.0075	0.012	0.0075	0.012
						0.01568	0.012	0.008	0.012
						0.01	0.012		
						0.01	0.012		
						0.0075	0.012		
						0.01	0.012		
						0.01	< 0.012		
						0.01	< 0.012		
Ni	1.8	0.26	23.8	2.47	0.22	1.9737	0.19	2.5	0.2
				2.32	0.22	2.45	0.154	1.991	0.217
				2.04	0.2	2.19	0.16	2.2	0.199
						1.6	0.13	1.72	0.16
						1.79	0.13	1.49	0.1302
						2.3	0.24	1.644	0.173
						2.49	0.26		
						2.17	0.19		
						2.69	0.21		
						2.22	0.19		
						2.308	0.236		
						2.34	0.23		
U	0.074	0.158	7	0.29	1.2	0.246	1.03	0.284	1.03
				0.3312	1.02	0.197	0.877	0.2909	0.8332
				0.3401	1.0238	0.22164	0.83	0.291	0.9292
						0.226	0.877	0.317	1.4
						0.268	1.22	0.274	0.953
						0.12261	1.72	0.247	0.857
						0.2382	1.53		
						0.22246	1.49		
						0.2723	1.45		
						0.212	1.3		
						0.254	1.47		
						0.2762	1.623		
Ca	195	19.6	434	195	16.5	220	14.6	184.36	14.43
				213.46	17.8	316	25.9	151	12.24

Ca				195	16.19	225	15.87	159.5	12.9
						199.5	12,8	233	16.4
						188	12.27	255	17.3
						264.2	20.2	239.1	17.439
						238	16		
						250	16.93		
						252	17.99		
						213	16.7		
						236	16.8		
						308	24.78		
K	15	25	1033	31	< 23	36	23	31.9	23
				37	< 23	41	23	32	23
				26	< 23	37	23	36	23
						24	23	32	23
						30	23	35	23
						57	23	35	23
						49.6	23		
						50	23		
						57	23		
						46	23		
						42	23		
						59	23		
Mg	187	19.391	681	278	22.3	291	18.15	291	20.94
				287	23.2	348.33	27.03	270	19.68
				244	19.1	304.72	20.63	266	19.75
						217	12.842	246.8	16.7
						239.87	14.527	221	13.63
						307	20.1	245	16.07
						330.4	21.1		
						315	20.13		
						397	26.9		
						317.9	22.592		
						340.22	22.41		
						342	25.33		
Mn	9.81	0.95	24	15.025	1.12	14.12	0.81	15	0.95
				15.097	1.0948	16.6	1.15	13.669	0.86
				12.9	0.9	14.8	0.898	13.72	0.9
						9.79	0.52	11.26	0.64
						11.05	0.6	9.662	0.56
						14.27	0.93	11.03	0.64
						16.078	0.97		
						15.49	0.862		
						19.04	1.09		
						15.11	0.972		
						16.1	0.97		
						15.729	1.01		
S	250	34.1	-	275	109	227	81	307	135
				252	116	250	86	246.44	101
				250	112	237	90	245	105
						215	78	230	91.6
						200	71	179	68.7
						227	75	195.9	71
						257	86		
						234.2	75		
						305	117		
						225	82.1		
						262	94		
S						230	94		

Zn	1.35	0.75	24.76	1.95	< 0.4	1.8	0.4	2.2	0.4
				1.78	< 0.4	2.175	0.4	1.7	0.4
				1.57	< 0.4	1.8	0.4	1.663	0.4
						1.459	0.4	1.64	0.4
						1.6	0.4	1.52	0.4
						2.27	0.4	1.5	0.4
						2.5	0.4		
						2.27	0.4		
						4.35	0.4		
						2	0.4		
						1.97	0.4		
						2	0.4		

Tab. A6: **Data of non-essential and essential elements of heap material in 50 cm depths**. Legend: O I/II/total – original soil fraction I, fraction II and total extraction of fraction I-VII after Zeien and Brümmer. I – mobile bioavailable, unspecific absorbed fraction; II – easy deliverable fraction. C I/II – control soil fraction I and II. M I/II – with mycorrhiza fraction I and II. P I/II – plants without mycorrhiza inoculation fraction I/II.

µg/g	**O I**	**O II**	**O total**	**C I**	**C II**	**M I**	**M II**	**P I**	**P II**
Al	105.4	6.15	81293	187	< 6	150	6	155.6	6
				193.6	< 6	132	6	157	6
				190.6	< 6	117.68	6	160	6
						87	6	117	6
						114	6	133.2	6
						101	6	129	6
						143.6	6		
						121.51	6		
						126	6		
						102	6		
						81.4	6		
						92	6		
Cs	0.0906	0.015	3.33	1.137	0.15	1.256	0.161	0.77	0.09663
				1.017	0.14	0.85	0.101	0.88	0.112
				1.06	0.15	0.7253	0.08476	1.14	0.15
						0.407	0.0394	0.53	0.0667
						0.72	0.082	0.8964	0.1079
						0.773	0.0982	0.839	0.111
						0.7	0.0928		
						0.59	0.074		
						0.58	0.074		
						0.651	0.083		
						0.646	0.0826		
						0.603	0.076		
Pb	0.08182	0.04	29.37	0.032	0.012	0.045	0.012	0.099	0.0322
				0.049	0.0131	0.084	0.02	0.096	0.022
				0.05	0.012	0.095	0.025	0.047	0.0131
						0.245	0.05402	0.04	0.012
						0.082	0.0203	0.0476	0.012
						0.042	0.012	0.0692	0.012
						0.057	0.0146		
						0.097	0.022		
						0.087	0.0183		
						0.02197	0.012		
						0.0179	0.0358		
						0.019	0.012		
Sr	0.719	0.105	131.4	0.7986	0.0967	1.099	0.12	0.9278	0.1099

				0.853	0.108	1.1859	0.119	0.875	0.105
				0.8539	0.112	1.093	0.105	0.931	0.09795
						1.001	0.09	1.361	0.141
						0.936	0.095	1.163	0.1418
						1.061	0.105	1.282	0.1358
						1.3125	0.142		
						1.668	0.173		
						1.23719	0.125		
						1.3158	0.13833		
						1.665	0.156		
						1.241	0.121		
Cd	0.00519	0.004	0.37	0.0075	< 0.012	0.012	0.012	0.0075	0.012
				0.01	< 0.012	0.0075	0.012	0.0075	< 0.012
				0.012	< 0.012	0.0075	0.012	<0.0075	< 0.012
						0.008	0.012	0.01	0.012
						0.01	0.012	0.0085	0.012
						0.011	0.012	0.00774	0.012
						0.0075	0.012		
						0.0075	0.012		
						0.01	0.012		
						0.0075	0.012		
						0.01134	0.012		
						0.008	0.012		
Ni	1.8	0.26	23.8	1.91	0.21	2.49	0.2	1.92	0.2
				2	0.21	1.89	0.16	2.2	0.172
				1.94	0.22	2.17	0.19438	2.42	0.2377
						1.24	0.13	1.7	0.12
						1.57	0.19	1.84	0.17
						1.87	0.16	1.84	0.2
						2.3	0.18		
						1.947	0.2		
						2.161	0.21		
						1.8	0.16		
						1.966	0.22		
						1.86	0.19		
U	0.074	0.158	7	0.301	0.799	0.215	0.918	0.2848	0.775
				0.297	0.964	0.257	0.806	0.346	1.005
				0.3276	0.8986	0.1909	0.918	0.384	1.24
						0.202	0.652	0.257	1.29
						0.22	0.78	0.389	1.5335
						0.2686	0.918	0.257	0.899
						0.305	1.32		
						0.262	0.91		
						0.251	0.81		
						0.199	0.665		
						0.13	1.027		
						0.2111	0.647		
Ca	195	19.6	434	195.04	16	248	19.2776	183	14.2
				219	19.8	211.5	14.6	227	17.26
				212	19.7	220	14.8	251	20
						203	11.7	273	19.76
						210	14.9	231	16.72
						251	18.81	263	20.55
						275	21.3		
						241	19.17		
						279	20.6		
						267	21.1		
Ca						348	25.6		

						287	19.6		
K	15	25	1033	32	< 23	45	23	20	23
				37	< 23	25.222	23	31	23
				35	< 23	27	23	25	23
						21	23	35	23
						25	23	37	23
						36.1	23	35	23
						37	23		
						30	23		
						27	23		
						32	23		
						36	23		
						32	23		
Mg	187	19.391	681	242	18.557	328	24.1	240	17.61
				258	22.2	242.3	15.83	277.1	19.95
				243.6	21.51	276	17.39	300.5	23.06
						180.6	10.152	251	17.24
						228	15.3	276	19.08
						270.8	19.08	265	19.82
						307	22.33		
						262	19.46		
						279	19.61		
						247	18.7263		
						272	18.9		
						267	17.8		
Mn	9.81	0.95	24	12.47	0.82	16.005	1.09	12.273	0.798
				13.32	1.05	11.64	0.67	14.17	0.84
				12.72	0.97	13.59	0.79	14.97	0.94
						7.77	0.45	11.342	0.687
						10.4	0.6	12.5	0.8
						12.32	0.75	11.96	0.7856
						14.76	0.92		
						12.841	0.86		
						13.7	0.86		
						11.7	0.78		
						12.64	0.86		
						12.56	0.79		
S	250	34.1	-	217	93	247	86	256	97
				237	100	197	67	289.6	112
				227	101	233.2	76	285	109
						181.3	56	215	82
						217.9	71	222	81
						245	94	210	82
						267	113		
						240	84		
						289	105		
						224	105		
						225	79		
						219	64		
Zn	1.35	0.75	24.76	1.47	< 0.4	2.3	0.4	2	0.4
				1.7	< 0.4	1.6	0.4	2.07	0.4
				1.72	< 0.4	1.9	0.4	2.9	0.4
						1.22	0.4	1.774	0.4
						1.32	0.4	1.7	0.4
						1.7	0.4	1.8	0.4
						2.25	0.4		
						5.2	0.4		
						1.84	0.4		

Zn						1.57	0.4		
						2.3	0.4		
						1.44	0.4		

Tab. A7: **Data of non-essential and essential elements of loess loam in 3 cm depths**. Legend: O I/II/total – original soil fraction I, fraction II and total extraction of fraction I-VII after Zeien and Brümmer. I – mobile bioavailable, unspecific absorbed fraction; II – easy deliverable fraction. C I/II – control soil fraction I and II. M I/II – with mycorrhiza fraction I and II. P I/II – plants without mycorrhiza inoculation fraction I/II.

µg/g	**O I**	**O II**	**O total**	**C I**	**C II**	**M I**	**M II**	**P I**	**P II**
Al	2.5	6	51082	4	6	4	6	4	6
				< 4	< 6	4	6	< 4	< 6
				< 4	< 6	4	6	< 4	< 6
Cs	0.0594	0.014	5.18	0.0295	0.0049	0.055	0.0083	0.053	0.0086
				0.032	0.0051	0.03866	0.007	0.054	0.0085
				0.049	0.008	0.049	0.007	0.035	0.0068
Pb	0.025	0.105	17.76	0.0075	0.0945	0.0075	0.087	0.0075	0.1073
				0.0075	0.0991	0.0075	0.084	0.0075	0.1064
				0.0075	0.112	0.0075	0.0867	0.0075	0.0958
Sr	13.163	2.92	116.5	26.25	3.7	29	4.28	25.07	3.382
				26.86	3.96	28.65	4.6	28.05	4.2
				27.9	3.8277	27.5	4.304	25.2	3.7
Cd	0.0115	0.2	0.72	0.0075	0.08	0.0075	0.07	0.0075	0.06
				0.0075	0.07	0.0075	0.064	0.0075	0.052
				0.0075	0.067	0.0075	0.06	0.0075	0.0651
Ni	1.37	5.35	94.82	0.19	1.79	0.12	1.61	0.16	1.534
				0.2	1.7	0.12	1.58	0.1	1.27
				0.202	1.7	0.138	1.65	0.104	1.46
U	0.041	4.606	17.53	0.158	1.294	0.243	1.173	0.157	1,1
				0.1901	1.2147	0.212	1.312	0.23	0.981
				0.2206	1.24	0.165	1.465	0.158	1.116
Ca	4478	1656.3	7955	5016	1703	5579,1	1863	4980	1750
				5199	1832	5734	1920	5506	2067.5
				5675	1679	5294	1736	4908	1717
K	65	25	16102	113	23	85	23	93	23
				110.7	23	95	23	107	23
				127	23	102	23	87	23
Mg	903	757.5	7020	251.46	854	210	888	247.2	898
				235.07	868	213	881	208	988
				259.7	806	216.6	835	242	828.88
Mn	1.72	30.75	540	1.72	52.1	0.98	52.23	1.9	47.4
				1.053	45.3	0.89	56.36	1.35	48.8
				1.85	53.2	2.32	58.83	1.5	48.2
S	1158	127	-	99	16	140	19	89.4	18
				88	26	147	26	86	15
				257	26	127	19.337	82	11
Zn	0.5	2.2	94.6	0.25	1.09	0.25	0.82	0.25	1.1
				0.25	0.83	0.25	0.71	0.25	1
				0.25	1.3	0.25	0.7	0.25	1.01

Tab. A8: **Data of non-essential and essential elements of loess loam in 50 cm depths**. Legend: O I/II/total – original soil fraction I, fraction II and total extraction of fraction I-VII after Zeien and Brümmer. I – mobile bioavailable, unspecific absorbed fraction; II – easy deliverable fraction. C I/II – control soil fraction I and II. M I/II – with mycorrhiza fraction I and II. P I/II – plants without mycorrhiza inoculation fraction I/II.

µg/g	O I	O II	O total	C I	C II	M I	M II	P I	P II
Al	2.5	6	51082	< 4	< 6	4	6	4	6
				< 4	< 6	< 4	< 6	< 4	< 6
				< 4	< 6	< 4	< 6	< 4	< 6
Cs	0.0594	0.014	5.18	0.061	0.0079	0.033	0.006	0.06	0.0086
				0.04	0.00603	0.05508	0.0082	0.025	0.0057
				0.055	0.0086	0.067	0.009	0.0554	0.00824
Pb	0.025	0.105	17.76	0.0075	0.118	0.0075	0.088	0.0075	0.1134
				0.0075	0.1126	0.0075	0.09	0.0075	0.097
				0.0075	0.1053	0.0075	0.09604	0.0075	0.1034
Sr	13.163	2.92	116.5	30	4.32	26.9	3.842	25.9	3.47
				32.8	5.952	28.7	4.16	22.5	3.01
				31.4	4.83	27.5	3.78	26.2	3.423
Cd	0.0115	0.2	0.72	0.0075	0.08	0.0075	0.06	0.0075	0.075
				0.0075	0.067	0.0075	0.06	0.0075	0.0791
				0.0075	0.067	0.0075	0.067	0.0075	0.082
Ni	1.37	5.35	94.82	0.195	2.1	0.13	1.62	0.16	2.1
				0.11	1.42	0.2	2.02	0.19	2.1
				0.17	1.8	0.19	2.255	0.19	1.932
U	0.041	4.606	17.53	0.21	1.472	0.163	1.35	0.217	1.76
				0.2756	1.11	0.1681	2.447	0.139	2.22
				0.242	1.27	0.245	1.8553	0.183	1.54
Ca	4478	1656.3	7955	5795	1881	5135	1618	5083	1573
				6471	1969	6118	1896	4429	1465
				6173	1919	5653	1743	4941	1585
K	65	25	16102	84	23	72	23	80	23
				82.4	23	85	23	64.3	23
				81	23	87	23	81	23
Mg	903	757.5	7020	269	887	219	789	290	797
				263.9	911	240.53	874	255.025	701
				247.8	910	244	854	293	826.3
Mn	1.72	30.75	540	1.27	56.2	0.43	39.9	0.72	43.751
				0.709	43.8	0.59	49.7	0.222	40.77
				1.03	49.5	1.37	55.4	0.93	45.61
S	1158	127	-	215	26	147	15	138	19
				393	28.8	729	56	216	30.7
				517	29.3	222	24.7	202	16.1
Zn	0.5	2.2	94.6	0.25	1.09	0.25	0.9	0.25	0.96
				0.25	0.7	0.25	1	0.25	1.09
				0.25	1	0.25	0.82	0.25	0.887

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8. Declaration (Selbstständigkeitserklärung)

Hereby I do declare, that this present work was independently written by myself and no other than indicated tools were used. All text passages, which comply in wording or sense other works, I have marked with references.

Hiermit erkläre ich, dass die vorliegende Arbeit eigenständig verfasst wurde und keine anderen als die angegebenen Hilfsmittel verwendet wurden. Alle Stellen, die anderen Werken im Wortlaut oder Sinn entsprechen, habe ich mit Quellenangaben kenntlich gemacht.

Steffi Gabriele Evelyn Formann

Jena, April 2016

9. Publications

Bizo ML, Formann S, Krause K, Roşu C, Kothe E (2013) Resistance of young mycorrhizal trees stresses caused by heavy metals such as Cs and Cd. *Environ Engin Manag J* 12:325-330.

Bizo ML, Nietzsche S, Mansfeld U, Langenhorst F, Majzlan J, Ozunu A, Formann S, Krause K, Kothe E (2016) Defense against lead pollution: mycorrhizal trees form the biomineral pyromorphite in roots and needles. *submitted*

Formann S, Krause K, Kothe E (2016) Benefit of guttation as adaptation strategy of fungi. *In preperation*

Formann S, Burow K, Nebelung K, Büchel G, Kothe E (2016) Bioaccumulation of radionuclides: from groundwater to food chains. *In preperation*